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Research Article

TSH receptor signaling via cyclic AMP inhibits cell surface degradation and internalization of E-cadherin in pig thyroid epithelium

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Abstract. Incorporation of E-cadherin into the adherens junction is a highly regulated process required to establish firm cell-cell adhesion in most epithelia. Less is known about the mechanisms that govern the clearance of E-cadherin from the cell surface in both normal and pathological states. In this study, we found that the steady-state removal of E-cadherin in primary cultured pig thyroid cell monolayers is slow and involves intracellular degradation. Experimental abrogation of adhesion by a Ca^{2+} switch induces rapid cell surface proteolysis of E-cadherin. At the same time, endocytosed intact E-cadherin and newly synthesized E-cadherin accumulate in intracellular compartments that largely escape further degradation. Acute stimulation with thyroid-stimulating hormone (TSH) or forskolin prevents all signs of accelerated E-cadherin turnover. The findings indicate that TSH receptor signaling via cyclic AMP stabilizes the assembly and retention of E-cadherin at the cell surface. This suggests a new mechanism by which TSH supports maintenance of thyroid follicular integrity.

Key words. E-cadherin; endocytosis; proteolysis; precursor; thyroid.

The adherens junction (AJ) brings cohesive strength to epithelial cells through a number of protein interactions. Adhesion is provided by E-cadherin, a transmembrane glycoprotein that homotypically binds to complementary E-cadherin molecules at the surface of adjacent cells. Intracellularly, the cytoplasmic tail of E-cadherin is directly associated with a group of regulatory proteins called catenins, which mediate a functionally important link to the submembranous actin-based cytoskeleton [1, 2]. The cadherin-catenin complex not only establishes firm cell-cell adhesion in mature epithelia, but also has a master role in conducting a variety of intracellular signals that together determine epithelial formation and behavior both in embryonic development and adult tissues [3, 4].

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The assembly of E-cadherin into a stable adhesion complex that builds up the AJ is a highly regulated process [reviewed in ref. 5]. E-cadherin is synthesized as a 135-kDa precursor polypeptide that is processed in the endoplasmic reticulum and the Golgi complex to its mature size of 120 kDa. At the cell surface, E-cadherin forms lateral (cis) and adhesive (trans) dimers [6] that require binding of Ca^{2+} in the extracellular N terminal of the molecule [7–9]. This process further concentrates and locks E-cadherin in a restricted portion of the plasma membrane that forms the AJ. Once integrated in the AJ, the turnover of E-cadherin is probably very low [10]. However, increased turnover of E-cadherin takes place when dynamic changes in adhesion are required, e.g. in cell migration and during epithelial-mesenchymal transition. However, knowledge of mechanisms that on the one hand prevent premature degradation of E-cadherin and on the

other govern the normal clearance of E-cadherin from the cell surface is sparse. The natural breakdown of E-cadherin in the steady-state probably takes place in lysosomes preceded by a ubiquitination process [11]. Endocytosed E-cadherin was recently shown to recycle to the cell surface [12], suggesting a mechanism that could rapidly modify the amount of preformed E-cadherin available for cell-cell adhesion.

The fact that E-cadherin requires Ca^{2+} to stay in a favorable conformation has frequently been employed to alter E-cadherin-mediated adhesion experimentally [13–15]. By reducing the extracellular Ca^{2+} concentration to micromolar levels, commonly referred to as the 'Ca^{2+} switch', the E-cadherin complex is rapidly dissociated and internalized leading to loss of adhesion and secondary disassembly of the entire junction complex [7, 16–20]**.** Despite the widespread use of Ca^{2+} switch experiments to explore the properties of E-cadherin in a cellular context, it is largely unknown if low Ca^{2+} treatment preferentially provokes degradation or recycling of E. cadherin.

In the present study, these aspects were investigated in primary cultured pig thyrocytes grown in bicameral chambers to mimic the organization of the natural epithelium. We found that reduction of extracellular Ca^{2+} rapidly induces proteolytic cleavage of E-cadherin, which takes place at the cell surface rather than in cytoplasmic compartments. Moreover, in the absence of Ca^{2+}-dependent adhesion, newly synthesized E-cadherin is partly processed, but resides intracellularly together with endocytosed mature E-cadherin that escapes further proteolysis. We also found that thyroid-stimulating hormone (TSH), the main regulator of thyroid differentiation, rapidly stabilizes E-cadherin at the cell surface and prevents, via a cyclic AMP (cAMP)-dependent mechanism, the accelerated turnover of E-cadherin.

Materials and methods

Cell culture and Ca^{2+} switch experiments

Isolation of thyroid follicles from pig and subsequent culture have previously been described in detail [21]. In short, segments of ruptured follicles were suspended in minimal essential medium (MEM) (Gibco, Paisly, UK) supplemented with 5% fetal calf serum, penicillin (200 U/ml; Gibco), streptomycin (200 µg/ml, Gibco Ltd) and fungizone/amphotericin (2.5 µg/ml, Gibco Ltd), and plated at a density of ~50 follicles/mm^2 on Transwell filters (pore size 0.4 µm) (Corning Costar Europe, Badhoevedorp, The Netherlands) precoated with collagen S (type I, 0.3 mg/ml; Roche Diagnostics Scandinavia, Bromma, Sweden). A tight and confluent monolayer, estimated by measuring the transepithelial resistance with a Millicell ohmmeter (Millipore, Bedford, Mass.), was established after culture for 6–7 days at 37 °C in 5% CO_2.

Extracellular Ca^{2+} was lowered by replacing the culture medium with serum- and Ca^{2+}-free MEM (Gibco) supplemented with 1.8 µM $CaCl_2$. As this treatment decreased the transepithelial resistance only slowly, the cells were initially exposed to Ca^{2+}-free MEM containing 1 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N′-tetraacetic acid (EGTA; Sigma-Aldrich, St. Louis, Mo.) for 10 min. Other treatments were 1 mU/ml TSH, 50 µM forskolin, 500 nM bafilomycin, 0.1 mM chloroquine, 10 µM brefeldin or 50 µg/ml cycloheximide (all from Sigma-Aldrich).

Immunofluorescence

Cells were fixed in 4% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.2, for 20 min at room temperature, followed by two rinses in phosphate-buffered saline (PBS) and permeabilization with 0.1% Triton X-100 in PBS for 5 min. After three washes in PBS, the cells were incubated with avidin-biotin blocking reagents (Vector Laboratories, Burlingame, Calif.) for 2 × 10 min and with blocking buffer (5% fat-free milk, 0.1% gelatine and 7.5% sucrose in PBS) for 5 min. Cells were incubated with primary antibodies against the C terminus of E-cadherin (clone 36, mouse monoclonal IgG; BD Transduction, Lexington, Ky.), the C terminus of human β-catenin (clone 14, mouse monoclonal IgG; BD Transduction) and occludin (rabbit polyclonal IgG; Zymed Laboratories, San Francisco, Calif.) for 1 h. Immunoreactivity was detected with biotin-conjugated secondary antibodies (Amersham Pharmacia Biotech, Buckinghamshire, UK) and fluorescein-isothiocyanate (FITC)-conjugated streptavidin (Amersham Pharmacia Biotech) incubated for 30 min each. Specificity was controlled by omitting primary antibody or incubating with irrelevant antibodies against non-junctional proteins. Filters with immunolabeled cells were placed on slides, mounted with Vectashield (Vector Laboratories) and examined in a Nikon Microphot FXA epifluorescence microscope equipped with a QLC100 confocal laser scanning module (VisiTech, Sunderland, UK). Images were captured on a digital camera and further processed using Image Pro Plu software (Media Cybernetics, Silver Spring, Md.)

Cell surface biotinylation

Cells were rinsed once in serum-free MEM and twice in ice-cold PBS, pH 7.5, and then incubated with 1.0 mg/ml sNHS-ss-biotin (Pierce Biotechnology, Rockford, Ill.), dissolved in PBS and added to both the apical and basal sides of the Transwell filter, for 30 min on a rocking platform on ice. Biotinylation was stopped by washing twice in PBS containing 100 mM glycine and twice in PBS for altogether 35 min. Cells were solubilized in lysis buffer

consisting of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Tx-100 and a mixture of protease inhibitors: 5 µg/ml each of leupeptin and aprotinin (Sigma-Aldrich) and 0.4 mM 4-(2-aminoethyl)-benzolsolfonyl-fluoride (Pefabloc; Roche Diagnostics Scandinavia). Streptavidin-agarose slurry (50% dissolved in PBS; Pierce Biotechnology) was then added and the lysates were incubated overnight at 4 °C with end-over-end rotation. Precipitated proteins were washed three times in lysis buffer, twice in high-salt buffer (lysis buffer with 500 mM NaCl) and once in Tris-HCl, pH 7.5. Each washing step was followed by centrifugation for 3 min at 350 g and, before addition of sample buffer, for 1 min at 9000 g. The samples were boiled for 4 min and precipitated proteins were analyzed with SDS-PAGE and Western blotting as described.

Western blot analysis

Cells were solubilized in a lysis buffer consisting of 0.5 M Tris-HCl, pH 6.8, 2% SDS and protease inhibitors. The protein concentration was determined with the Micro BCA protein assay kit (Pierce Biotechnology). Samples of equal protein concentrations were boiled for 4 min and subjected to SDS-PAGE using 4–15% polyacrylamide Readygels (Bio-Rad, Upplands-Väsby, Sweden), after which the separated proteins were transferred by electroblotting to nitrocellulose filters (pore size 0.45 µm) in a mini-transblot cell (Bio-Rad). The blots were preincubated for 1 h in blocking buffer consisting of 5% dry milk in TBS-Tween (20 mM Tris-HCl, 137 mM NaCl and 0.1% Tween 20, pH 7.6) and then incubated with primary antibodies against E-cadherin, β-catenin and occludin and with secondary horseradish peroxidase-conjugated antibodies (Dako, Glostrup, Denmark) for 1 h each. Immunolabeled proteins were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) according to the manufacturer's instructions. Protein mass was calculated by comparison to prestained SDS-PAGE molecular-weight standards (Bio-Rad). Densitometric evaluation of immunoblotted proteins was performed on a GS-700 imaging densitometer (Bio-Rad) using the Molecular Analyst Software.

Metabolic labeling, immunoprecipitation and autoradiography

Cells were washed with serum-free MEM devoid of leucine (MEM-leu; Gibco) and incubated with 100 µCi/ml [^{3}H]leucine (Amersham Pharmacia Biotech) in MEM-leu added to the bottom Transwell chamber, for 4 h at 37 °C. After washing in radioactivity-free MEM, the cells were either fixed and further washed in 10% trichloroacetic acid for quantification of total protein-bound radioactivity by liquid scintillation, or subjected to immunoprecipitation of E-cadherin. For this purpose, cells were solubilized in lysis buffer (0.5% Triton X-100, 0.01 M maleinic acid, 0.13 M NaCl, 0.03 M KCl, 5 mM glucose, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 0.01 M Tris, pH 6.8) for 20 min in the presence of protease inhibitors. The solutes were diluted to equal protein concentrations and incubated with anti-E-cadherin monoclonal antibody (mAb) for 1.5 h at 4 °C, after which protein A-Sepharose CL4-B (Amersham Pharmacia Biotech) was added and further incubated for 1 h on a rocking platform. After centrifugation at 10,000 g, the immunoprecipitates were dissolved in sample buffer, heated to 96 °C for 4 min, and separated by electrophoresis in a 4–20% polyacrylamide gradient gel (Mini-Protean II; Bio-Rad). Finally, the gels were impregnated with Amplify (Amersham Pharmacia Biotech) and exposed to autoradiographic film (Hyperfilm; Amersham Pharmacia Biotech).

Results

Redistribution of E-cadherin in pig thyrocytes after switch to low extracellular Ca^{2+}

A Ca^{2+} switch protocol was employed to rapidly reduce extracellular Ca^{2+} to micromolar levels and thereby dissociate Ca^{2+}-dependent cell-cell adhesion. Similar to earlier observations [17], this treatment caused a gradual loss of E-cadherin from the thyroid cell surface likely due to internalization, and after 4 h most E-cadherin had obtained a perinuclear cytoplasmatic distribution (fig. 1A, B). At the same time, β-catenin normally bound to E-cadherin showed a similar route of translocation to the cell interior (fig. 1C, D). The tight-junction protein occludin was dislocated from the plasma membrane after incubation in low-Ca^{2+} medium (fig. 1E, F), indicating that the provoked redistribution was not restricted to the cadherin-catenin complex. The following experiments were conducted to investigate if the accelerated endocytosis of E-cadherin was accompanied by increased degradation.

Degradation and internalization of E-cadherin in low-Ca^{2+} conditions

Confluent cells grown in normal Ca^{2+} medium predominantly expressed the 120-kDa mature form of E-cadherin, but small amounts of low-molecular-weight fragments of approximately 110, 85, 55 and 35 kDa were also detected (fig. 2A, lane 1). Biotinylation experiments indicated that most if not all intact E-cadherin was present at the cell surface whereas the presumed degradation products were located in the cytoplasm (fig. 2A, lane 2). In Ca^{2+}-depleted cells, the total level of E-cadherin was slightly reduced (fig. 2A, lane 3). However, in conformity with the immunofluorescent findings, E-cadherin gradually disappeared from the cell surface and after 4 h in low Ca^{2+} most of the remaining mature E-cadherin had accumulated intracellularly (fig. 2A, lane 4, fig. 2B). At the same

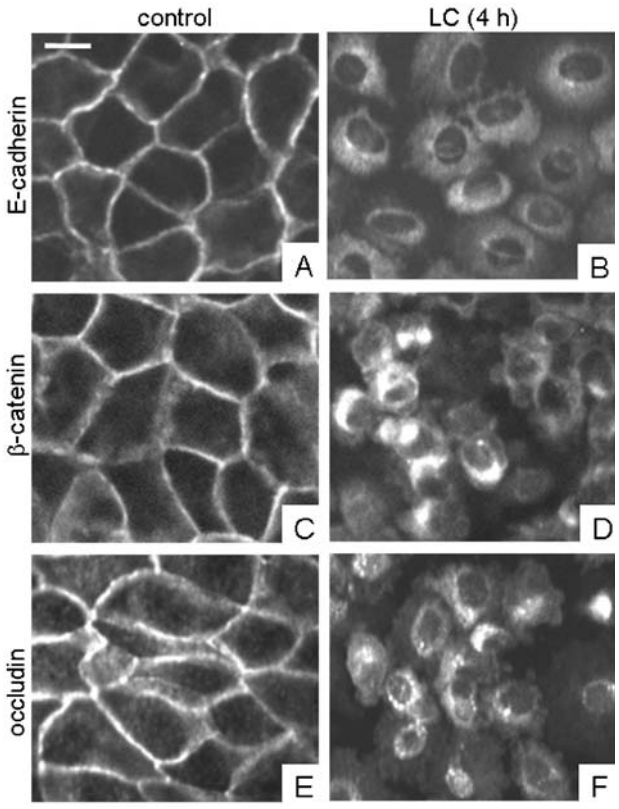

Figure 1. Redistribution of E-cadherin, β-catenin and occludin in filter-cultured pig thyrocytes after switch to a low Ca^{2+} concentration. E-cadherin, β-catenin and occludin were mainly located at the cell-cell contacts in untreated cells (*A*, *C*, *E*), while all three junction-associated proteins showed a perinuclear staining in cells exposed to 1.8 µM Ca^{2+} for 4 h (*B*, *D*, *F*). No immunoreactivity was observed at the cell surface. Scale bar, 10 µm.

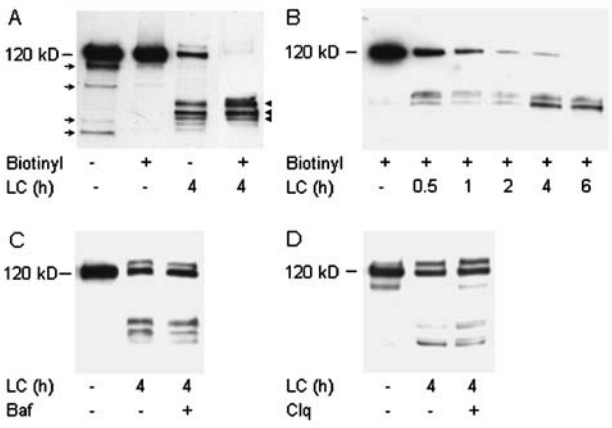

Figure 2. Altered degradation of E-cadherin in low-Ca^{2+} conditions. (*A*) Western blot analysis of whole-cell lysates (lanes 1 and 3) and cell surface biotinylated samples (lanes 2 and 4). Mature E-cadherin (120 kDa) and degradation products are indicated (110, 85, 55 and 35 kDa by arrows and 78, 70 and 68 kDa by arrowheads). (*B*) Time-dependent loss of E-cadherin accessible to cell surface biotinylation after incubation in low-Ca^{2+} medium (LC). (*C*, *D*) Lack of effects of bafilomycin (Baf, 500 nM) and chloroquine (Clq, 0.1 mM) on E-cadherin degradation.

time, the E-cadherin fragments residing in the cytoplasm in untreated cells were lost and replaced by other cleavage products of 78, 70 and 68 kDa (fig. 2A, lane 3). Unexpectedly, these newly formed E-cadherin fragments were recovered by cell surface biotinylation (fig. 2A, lane 4). Thus, the fate of E-cadherin losing adhesion after reduction of the extracellular Ca^{2+} levels was twofold: one fraction was rapidly degraded, presumably at the cell surface, whereas another larger fraction was internalized without signs of being cleaved.

Endocytosed E-cadherin has recently been shown to recycle to the cell surface [12]. To exclude the possibility that the observed breakdown of E-cadherin in low-Ca^{2+} conditions took place intracellularly, and that the cleavage products are subjected to recycling by default, we investigated if the degradation could be blocked by inhibiting endosomal function. However, as the degradation pattern of E-cadherin was the same in Ca^{2+}-depleted cells simultaneously treated with bafilomycin (fig. 2C) or chloroquine (fig. 2D), endocytosis and endosomal degradation were likely not involved.

Synthesis and processing of E-cadherin after a Ca^{2+} switch

Another constant finding in Ca^{2+}-depleted cells was the appearance of a larger form of E-cadherin of approximately 135 kDa (fig. 2C, D). After prolonged incubation in low Ca^{2+} for 24 h, the amount of the 135-kDa form often exceeded that of the mature protein (fig. 3A). Blocking of Golgi transport by brefeldin in cells kept in normal medium also caused accumulation of the larger E-cadherin (fig. 3B). Moreover, treatment with cycloheximide prevented its appearance (fig. 3C, lanes 1 and 2). Together, these findings indicated that the 135-kDa form in all probability was identical to the E-cadherin precursor previously characterized [10]. To further support this notion, [^{3}H]leucine metabolic labeling experiments were conducted. This showed that total protein synthesis was not significantly different between controls and Ca^{2+}-depleted cells (data not shown), and that E-cadherin continued to be expressed in low Ca^{2+} conditions (fig. 3D). Also evident was that newly synthesized E-cadherin was processed to mature protein and some slightly smaller immunoreactive species (fig. 3D), the size of which did not correspond to those simultaneously generated by proteolytic cleavage of E-cadherin at the cell surface. As almost no mature E-cadherin could be detected at the surface of cells depleted of Ca^{2+} for 4 h (see fig. 2A, B), after processing the newly formed E-cadherin likely accumulated in the cytoplasm. It is however noteworthy that neosynthesis contributed little to the total amount of E-cadherin in Ca^{2+}-depleted cells (fig. 3C, lanes 1 and 2).

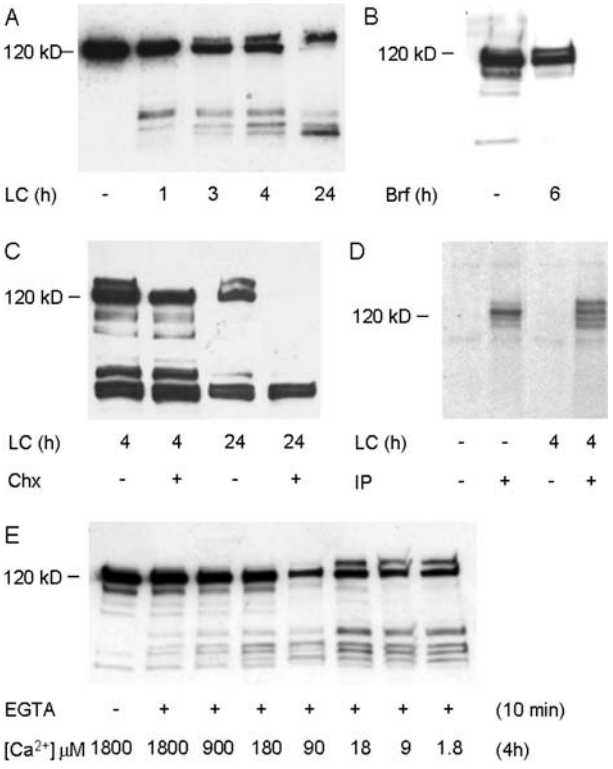

Figure 3. Accumulation of E-cadherin precursor after a switch to low-Ca^{2+} medium. (*A*) Time-dependent increase of a 135-kDa band that is immunoreactive to the E-cadherin antibody. (*B*) Brefeldin (Brf, 10 µM) caused 135-kDa E-cadherin accumulation and blocked E-cadherin degradation in normal Ca^{2+} conditions. (*C*) Cycloheximide (Chx, 50 µg/ml) added 1 h before the Ca^{2+} switch blocked the accumulation of E-cadherin precursor and the accelerated breakdown of mature E-cadherin in cells incubated in low Ca^{2+}. (*D*) Autoradiography of precursor and processed E-cadherin recovered by immunoprecipitation (IP) of [^{3}H]leucine-labeled cells. Note that minute amounts of the 135-kDa precursor and two large degradation bands were also present in cultures not subjected to low Ca^{2+}. (*E*) Different extracellular Ca^{2+} levels inducing cell surface degradation of mature E-cadherin and accumulation of E-cadherin precursor. All cultures with reduced extracellular Ca^{2+} levels were initially incubated with EGTA in Ca^{2+}-free medium for 10 min to rapidly dissociate the junction complex.

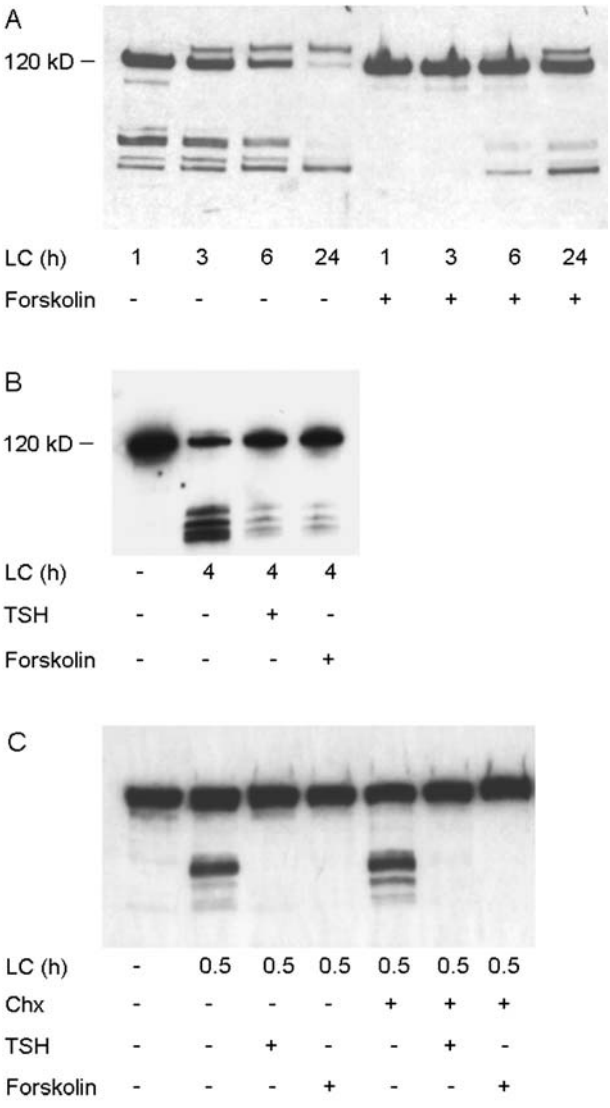

Figure 4. TSH and forskolin counteract the accelerated turnover of E-cadherin after the Ca^{2+} switch. (*A*) Time-dependent changes in E-cadherin (precursor, mature and degraded forms) following incubation in low-Ca^{2+}medium (LC) with or without forskolin (50 µM) for 1–24 h. (*B*) TSH (1 mU/ml) reproduced the protective effect of forskolin on E-cadherin degradation. (*C*) The protective effect of TSH (1 mU/ml) or forskolin (50 µM) was immediate (<30 min) and not affected by cycloheximide (Chx, 50 µg/ml) treatment (added 1 h before the Ca^{2+}-switch).

In fact, to fully replace the preformed E-cadherin pool with E-cadherin synthesized after the Ca^{2+} switch, prolonged incubation in low Ca^{2+} for up to 24 h was required (fig. 3C, lanes 3 and 4).

The different E-cadherin alterations provoked by the Ca^{2+} switch evidently had a common cause in the reduced extracellular Ca^{2+} level, but whether they were mechanistically related phenomena was not obvious. To explore this, E-cadherin was investigated in cultures subjected to gradually decreasing Ca^{2+} concentrations. As shown in figure 3E, altered proteolysis of E-cadherin was observed already after 10 min EGTA treatment (fig. 3E). However, accelerated endocytosis of E-cadherin (data not shown) and accumulation of the E-cadherin precursor (fig. 3E) was found only in cells incubated in medium with ≤18 µM Ca^{2+}. Altogether, this suggests that cell surface degradation of E-cadherin after the switch to low Ca^{2+} takes place independently of other changes in E-cadherin turnover.

Effect of TSH and forskolin on the turnover and localization of E-cadherin

From previous studies on the regulation of the thyroid epithelial barrier [22, 23] we know that TSH receptor activation of the cAMP signaling pathway effectively in-

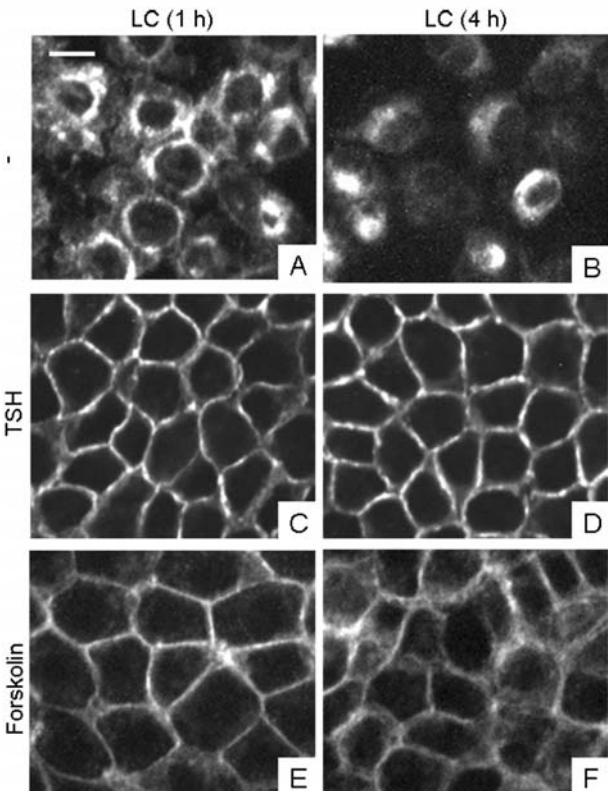

Figure 5. TSH and forskolin inhibit endocytosis of E-cadherin after the Ca^{2+} switch. Incubation in low Ca^{2+} resulted in redistribution of E-cadherin from the lateral membrane to the perinuclear cytoplasm already after 1 h (*A*), and the staining pattern remained for 4 h (*B*). The internalization of E-cadherin in low Ca^{2+} conditions was inhibited by acute stimulation with TSH (*C*, *D*) and forskolin (*E*, *F*); only a slight discontinuity of the E-cadherin distribution along the cell-cell contacts is observed. Scale bar, 10 µm.

hibits the junction-breaking effect of extracellular Ca^{2+} removal. Of interest, therefore, was to investigate if E-cadherin might be a target for the protection mechanism. As shown in figure 4A, forskolin was able to nearly fully block the degradation of E-cadherin in low Ca^{2+}, and the appearance of the 135-kDa precursor was also much delayed. Similar protective effects on E-cadherin were observed in TSH-stimulated cultures (fig. 4B). The protection was rapid and not affected by cycloheximide treatment (fig. 4C), but the inhibitory effect sustained when the Ca^{2+}-depleted state continued for as long as 24 h (fig. 4A). That increased TSH receptor signaling via cAMP actually stabilized the localization of E-cadherin at the cell-cell contacts was evident in immunofluorescent images (fig. 5 A–F); no signs of dissociation and internalization of E-cadherin were observed in Ca^{2+}-depleted cells that were co-stimulated with TSH or forskolin.

Discussion

Employing the Ca^{2+} switch technique we found in the present study that E-cadherin was rapidly degraded in primary cultured thyroid epithelial cells incubated in low-Ca^{2+} medium. In contrast to untreated cells, in which the slow E-cadherin turnover involved intracellular degradation, the accelerated proteolysis in Ca^{2+}-depleted cells occurred exclusively at the surface, generating several proteolytic fragments of E-cadherin that were retained in the plasma membrane for several hours. At the same time, a large pool of intact E-cadherin was gradually internalized without being degraded. This indicates that a subpopulation of surface-bound E-cadherin is highly susceptible to endogenous proteolytic attack once Ca^{2+} is removed from the extracellular domain. Based on the size of the retained cleavage products (68, 70 and 78 kDa), and the fact that immunoreactivity against an epitope located in the cytoplasmic tail of the molecule was preserved, the degradation of E-cadherin was likely mediated by an extracellular, presumably membrane-bound protease. The ectodomain of E-cadherin can be enzymatically cleaved, e.g. by metalloproteinases [24–26] and presenilin-1/g-secretase [27], during processes involving dynamic alterations of cell adhesion. However, neither of the E-cadherin fragments appearing in the Ca^{2+}-depleted thyrocytes corresponded to those previously reported in the literature [24–26, 28, 29], giving no clues as to the identity of the protease(s) involved.

Since the stability of E-cadherin homodimers is strictly dependent on the presence of Ca^{2+} [30], loss of homophilic binding likely makes E-cadherin susceptible for surface cleavage. In fact, this is very similar to the ability of E-cadherin to resist trypsin digestion unless adhesion is broken by Ca^{2+} removal, as originally described in Takeichi's classical experiments [31]. The multiple binding of calcium ions in the extracellular domain of E-cadherin has been shown to stabilize a conformation of the N terminal that favors homophilic adhesion [32]. Conversely, E-cadherin depleted of Ca^{2+} undergoes a structural modification from a rod-like to a more condensed shape along with a loss of the ability to dimerize [14]. Such a conformational change may thus expose protease-sensitive sites of the molecule that are otherwise hidden and protected from inappropriate cleavage. In vitro experiments on recombinant E-cadherin have further shown that the Ca^{2+}-binding pockets of the extracellular domain possess different Ca^{2+}-binding affinities [33]. Interestingly, we found here that E-cadherin was readily degraded when Ca^{2+} was modestly reduced down to 90 µM, whereas Ca^{2+} concentrations less than 18 µM were required to also induce endocytosis of E-cadherin and accumulation of the precursor. Beside adding further support to the notion that proteolytic cleavage affects not all but a distinct fraction of E-cadherin present at the cell

surface, this suggests that E-cadherin need not be fully dissociated to gain protease sensitivity.

TSH and, even more effectively, the cAMP-generating agent forskolin were found to prevent the degradation of E-cadherin in the Ca^{2+}-depleted thyrocytes. The fact that only a short stimulation period with TSH or forskolin was required to protect E-cadherin, and that protection could not be blocked by cycloheximide, indicates that a post-translational mechanism independent of gene expression was involved. Conceivably, when the cAMP signaling pathway is activated, E-cadherin is rapidly stabilized in a conformation that restricts Ca^{2+} depletion. This possibility is supported by earlier findings that neutralizing E-cadherin antibodies do not to dissociate E-cadherin binding, presumably because they fail to reach hidden epitopes, in forskolin-treated MDCK cells [34]. As there is no known cAMP-dependent kinase (PKA) consensus binding site in its cytoplasmic tail, E-cadherin is probably influenced indirectly. However, the cadherin-catenin complex constitutes a regulatory unit that modifies cadherin binding and adhesion in response to e.g. kinase activities [35–37]. Thus, reinforced cadherin-catenin interaction may be one possible mechanism by which the stabilizing effect of TSH on E-cadherin is transduced. Reduced dissociation of E-cadherin after Ca^{2+} depletion may also be accomplished by preventing the AJ-associated actin ring from being concomitantly retracted into the cytoplasm [38–40]. Interestingly, PKA was recently demonstrated to bind to IQGAP1 [41], an effector of the cytoskeletal regulator Rac1 that is known to impair adhesion by binding to β-catenin [42, 43]. The precise function of PKA in this interaction and the possible impact on cadherin-binding properties have, however, not yet been investigated. Whether the protective effect of TSH on E-cadherin degradation observed in this study is mediated by PKA also remains to be proven. In fact, some cAMP-mediated effects on junctional proteins may be PKA independent [44].

Agents (bafilomycin and chloroquine) known to inhibit prelysosomal endocytic transport had no effect on the accelerated degradation of E-cadherin in Ca^{2+}-depleted thyrocytes. On the contrary, the low levels of E-cadherin cleavage products present in the cytoplasm of untreated cells disappeared soon after the switch to low Ca^{2+}. This indicates that intracellular proteolysis did not contribute to the degradation of E-cadherin and was in fact blocked by the treatment. However, intact E-cadherin was readily internalized and accumulated in the cytoplasm, thus escaping both cell surface and lysosomal proteolysis. Internalized E-cadherin is likely, therefore, to have entered a distinct intracellular compartment that managed to avoid further routing to the endolysosomal system. This is strikingly similar to a recently characterized postendocytic compartment that contained junctional proteins (including E-cadherin) and was formed in the apical cytoplasm of T84 intestinal epithelial cells after Ca^{2+} removal [45]. Interestingly, this storage compartment displayed several basolateral membrane proteins but was devoid of markers of both early and late endosomes. A function for a unique endocytic pathway might be to save as much as possible of the mature E-cadherin pool for recycling when new AJs are to be formed. In this respect, an important finding in the present study was that stimulation with TSH and forskolin not only inhibited the degradation but also prevented the internalization of E-cadherin from the cell surface and the accumulation of E-cadherin precursor in the cytoplasm. This suggests that the intracellular transport of E-cadherin is under regulatory control by the cAMP signaling pathway.

In conclusion, our data indicate that the turnover of intracellular and cell-surface-associated E-cadherin is a tightly coordinated process subjected to hormonal regulation by TSH in cultured thyroid cells. A low degradation rate of E-cadherin presumably taking place in the endolysosomal system characterizes the epithelium that has established firm Ca^{2+}-dependent cell-cell adhesion. However, cell surface cleavage of E-cadherin predominates over intracellular proteolysis when the homotypic binding is rapidly broken by experimental removal of extracellular Ca^{2+}. Concomitantly, mature E-cadherin escaping the proteolytic attack is internalized and accumulated in a largely protease-resistant intracellular compartment; together with a constant influx of newly synthesized and processed E-cadherin this constitutes a cytoplasmic pool of E-cadherin that might potentially be used for reformation of the AJ. TSH via activation of the cAMP signaling pathway counteracts all these effects by stabilizing E-cadherin already present and integrated in the AJ complex. In a physiological context, TSH is therefore likely to promote maintenance of thyroid epithelial integrity and surveillance of the functionally important barrier between the follicle lumen and the extrafollicular space.

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