E-cadherin-dependent Transcriptional Control of Apolipoprotein A-IV Gene Expression in Intestinal Epithelial Cells

A ROLE FOR THE HEPATIC NUCLEAR FACTOR 4

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Cell-matrix and cell-cell adhesion play a central role in the control of cell proliferation, differentiation, and gene expression. Integrins and E-cadherin are the key components involved in these processes in epithelial cells. We recently showed that integrin-dependent adhesion to the extracellular matrix reinforces the formation of E-cadherin-actin complexes inducing the polarization of Caco-2 enterocytes and increases the expression of a marker of enterocyte differentiation, the apolipoprotein A-IV (apoA-IV) gene. By impairing or enhancing E-cadherin-dependent cell adhesion, we demonstrate in the present study its involvement in the transcriptional activation of the apoA-IV gene in Caco-2 cells. This control requires the regulatory sequence that we have previously identified as necessary and sufficient to drive and restrict apoA-IV gene expression in enterocytes in vivo. Furthermore, using chimeric E-cadherin-Fc homophilic ligand-coated surfaces, we show that a direct activation of E-cadherin triggers the transcriptional activation of the apoA-IV promoter. Finally, E-cadherin-dependent cell-cell adhesion controls the nuclear abundance of the transcription factor hepatic nuclear factor 4α, which is involved in the enterocyte-specific expression of apoA-IV gene. Altogether, our results suggest that E-cadherin controls enterocyte-specific expression of genes, such as the apoA-IV gene, through the control of hepatic nuclear factor 4α nuclear abundance.

Epithelial differentiation is an active process resulting from the integrated response to cues derived from the intrinsic gene program and the microenvironment (1–3), through a molecular cross-talk involving cell-extracellular matrix (ECM) and cell-cell adhesions (4). Connections are made via membrane-bound receptors and transmitted to the nucleus, where signals result in modifications of the nuclear matrix and chromatin structure (1, 5) and lead to selective gene expression (1, 3).

The functional maintenance of the intestinal epithelium displays specific features: a rapid and permanent renewal from stem cells located at a fixed position within the crypt, the migration of cells “en cohorte” along the crypt to villus axis, and a cell shedding at the apex of the villi (6). These features make especially important the contribution of cell adhesion systems to the control of intestinal epithelium differentiation, considering that cell adhesion to ECM varies during cell migration along with the gradual loss of stem cell properties and the acquisition of differentiated features (7).

ECM components are known to control cell proliferation and differentiation in many tissues through interactions with integrins (8, 9). They are cooperatively produced by mesenchymal and epithelial cells and are differentially distributed along the crypt-to-villus axis in both the developing and the adult intestine (10, 11), as is the integrin repertoire expressed by enterocytes (11, 12). The differential distribution of ECM molecules may depend on and, in return, modulate intestinal cell differentiation (13, 14).

The pattern of expression of E-cadherin, one of the major cell-cell adhesion molecules in the intestinal epithelium, also varies along the crypt-to-villus axis, with a lesser abundance in undifferentiated cells in the crypt (15). Cadherins mediate cell-cell adhesion through Ca²⁺-dependent homophilic interaction of their extracellular domain and anchoring of their cytoplasmic domain to the actin cytoskeleton by catenins (16, 17). E-cadherin-mediated cell-cell attachment plays an important role in the differentiation, polarization, and homeostasis of many epithelia (18, 19). Its importance in the renewal of the intestinal epithelium has been demonstrated in vivo (4). In cell models, E-cadherin down-regulation has been associated with various phenotypic alterations, including loss of cell polarity and increased cell proliferation, which can in turn prevent cell differentiation (20–23).

To investigate the mechanisms by which adhesion controls the onset of differentiated functions in enterocytes, we chose as a differentiation marker the apolipoprotein A-IV (apoA-IV) gene, the expression of which is restricted to differentiated enterocytes of the villi in the proximal small intestine (24). We have previously demonstrated that the apoA-IV promoter (~700 bp) was necessary and sufficient to restrict apoA-IV expression to villous enterocytes when fused with the apoC-III enhancer (24) (Fig. 1). A hormone-responsive element (HRE) located within the distal region of the apoA-IV promoter confers the physiological pattern of apoA-IV gene expression in the intestine (24, 25). The nuclear factor HNF-4α selectively binds to this HRE (25–27) and controls apoA-IV gene transcription in vivo and ex vivo (26, 27).

Using Caco-2 enterocytes (28), we have demonstrated that ECM is able to induce the expression of the apoA-IV gene at both protein and mRNA levels (29, 30). In parallel, cell-ECM interactions through integrins reinforce the cellular polarization and the cell-cell adhesion by...
targeting E-cadherin at the lateral membrane in functional complexes with actin cytoskeleton (30). These results suggested that the activation of E-cadherin might contribute to the control of apoA-IV gene expression.

The purpose of our present study was to investigate, by means of impairment and activation experiments, the involvement of E-cadherin-mediated cell-cell adhesion in the control of apoA-IV gene transcription in intestinal cells and the potential role of HNF-4α in this regulation.

**E-cadherin Controls ApoA-IV Gene Transcription**

For E-cadherin activation experiments, culture plates were precoated overnight at 4°C with an anti-Fc antibody (0.5 μg/cm² in PBS/Ca²⁺) (Jackson ImmunoResearch) and then coated with a recombinant human E-cadherin-Fc chimera (R&D Systems) (10 μg/cm² in PBS/Ca²⁺) for 2 h at room temperature followed by PBS/Ca²⁺ wash and blocked with PBS/Ca²⁺ containing 1.5% bovine serum albumin. Plates coated with anti-Fc antibodies only were used for controls. Then eC3A4-Luc cells were seeded at low density in these coated plates and cultured for 48 h.

For the actin cytoskeleton perturbation experiments, Caco-2 cells were seeded at low density, and at the time indicated in the results, the cells were treated with 0.5 or 2 μM of Latrunculin B for 48 h in serum-free medium.

**Reporter Gene Expression Analysis**—GFP expression was analyzed by flow cytometry or by confocal microscopy. For flow cytometry analysis, eC3A4-GFP cells were carefully trypsinized to obtain a single cell suspension. This suspension was rinsed in PBS and fixed for 1 h in 4% paraformaldehyde at 4°C. Cells were then centrifuged for 10 min at 400 × g, and the pellets were resuspended in PBS and kept at 4°C until analysis. Flow cytometry analysis was performed using a Coulter Altra cell sorter (Beckman-Coulter) equipped with an argon laser. The excitation wavelength was set at 488 nm, and the GFP emission was recovered at 510 nm. For each sample, 10,000 cells were analyzed. The results are expressed as the mean fluorescence values ± S.D. for triplicate cultures. For confocal analysis, eC3A4-GFP cells were grown on LabTek chambered borosilicate cover glass (Nunc). GFP fluorescence was quantified using software from a Zeiss LSM 510 confocal laser-scanning microscope.

For luciferase analysis, eC3A4-Luc cells were washed twice with PBS and kept at −80°C until extraction. The cells were thawed in the presence of the luciferase extraction buffer (25 mM Tris, pH 7.8, 8 mM

(100,000 cells/cm²) or low density (12,000 cells/cm²). The medium was changed every day.

For integrin perturbation experiments, the eC3A4-GFP cell suspension was incubated with a monoclonal anti-β1 integrin blocking antibody (6G6; Chemicon) (5 μg/ml) or a monoclonal anti-β1 integrin activating antibody (B3B11; Chemicon) (10 μg/ml) during 20 min at room temperature before being seeded in the presence of the antibodies at high density in culture plates that had or had not been coated with ECM. Purified mouse IgG (Chemicon) was used as a control. Cells were analyzed after 48 h for GFP fluorescence by confocal microscopy.

For E-cadherin perturbation experiments, Caco-2 cells or eC3A4-Luc cells were seeded at high density. For the calcium switch experiments, at the time indicated under "Results," cells were treated for 10 min with a solution of PBS containing 2 mM EGTA and 2.5 mM glucose and then placed for 48 h in a serum-free medium containing (+/+ or not containing (+/+ or not containing (+/−) or calcium. In some experiments, a blocking anti-E-cadherin antibody (SHE78-7; Takara), or nonspecific IgG (Chemicon), was added at a final concentration of 10 μg/ml. The cells were changed after 24 h of treatment with fresh serum-free medium containing or not containing calcium and containing or not containing SHE78-7 or nonspecific IgG.

For fully differentiated cells, low density Caco-2 cells were cultured for 16 days with normal calcium concentration. At day 16, cells were treated for 10 min with 2 mM EGTA, as described above, and then cultured for 48 h with fresh serum-free medium containing (+/+) or not containing (+/−) calcium, to disrupt or not disrupt calcium-dependent cell-cell junctions.

To grow cells in suspension, 850,000 eC3A4-Luc cells were suspended in 1.5 ml of medium described above, in 15-ml polypropylene tubes (Techno Plastic Products AG).

For E-cadherin activation experiments, culture plates were precoated overnight at 4°C with an anti-Fc antibody (0.5 μg/cm² in PBS/Ca²⁺) (Jackson ImmunoResearch) and then coated with a recombinant human E-cadherin-Fc chimera (R&D Systems) (10 μg/cm² in PBS/Ca²⁺) for 2 h at room temperature followed by PBS/Ca²⁺ wash and blocked with PBS/Ca²⁺ containing 1.5% bovine serum albumin. Plates coated with anti-Fc antibodies only were used for controls. Then eC3A4-Luc cells were seeded at low density in these coated plates and cultured for 48 h.

For the actin cytoskeleton perturbation experiments, Caco-2 cells were seeded at low density, and at the time indicated in the results, the cells were treated with 0.5 or 2 μM of Latrunculin B for 48 h in serum-free medium.

**Experimental Procedures**

** Constructs and Cell Transfection**—The eC3A4-GFP cells were obtained as follows. A plasmid (eC3A4-GFP) expressing the green fluorescent protein (E-GFP) under the control of the eC3-A4 regulatory sequences, which restrict the expression of the apoA-IV gene to villus enterocytes (24, 31), was constructed by cloning the −700/+10 apoA-IV promoter fused to the −890/−500 apoC-III enhancer into the SacI and HindIII sites of the promoterless vector pEGFP-1 (Clontech). Caco-2 cells (28, 31) were transfected with eC3A4-GFP or with the empty GFP plasmid (mock-transfected) as a control, using the DNA calcium phosphate precipitate method. Individual clones were isolated after 3 weeks in G418 (Invitrogen) (400 μg/ml) and further subcloned by the limit dilution method. Flow cytometry experiments showed that the GFP fluorescence signals obtained in mock-transfected and nontransfected Caco-2 cells were perfectly superimposed. After confluence, GFP was induced to similar levels in all clones obtained. The eC3A4-GFP-3 clone was used for further studies and was subsequently designated as eC3A4-GFP cells. The eC3A4-Luc cells have been previously described (26).

**Preparation and Coating of the Extracellular Matrix**—The native ECM was prepared as described previously (29, 30) from 3-day postconfluence HT29-MTX cells (HT29 cells that had been adapted to 10−5 M methotrexate (32, 33)).

**Cell Culture and Perturbation Experiments**—Caco-2, eC3A4-GFP, and eC3A4-Luc cells were cultured at 37°C in 10% CO2 in Dulbecco’s modified Eagle’s medium containing or not containing calcium supplemented with 25 mM glucose, 50 units/ml penicillin, 50 μg/ml streptomycin, 1% nonessential amino acids (Invitrogen), and 20% heat-inactivated fetal calf serum (AbCys). Cells were plated out at high density
E-cadherin Controls ApoA-IV Gene Transcription

MgCl₂, 1 mM EDTA, 15% glycerol, 1% Triton X-100, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride), scraped, and transferred into Eppendorf tubes and frozen and thawed twice. The cellular debris were eliminated by centrifugation, and the supernatant was used for luciferase activity measurement (34). Protein contents determination was made with the Bio-Rad DC protein assay.

Immunofluorescence Studies—Cells, grown on LabTek chambered borosilicate cover glass (Nunc), were fixed in 4% paraformaldehyde in phosphate-buffered saline. Non-specific antigens were blocked by incubating in PBS containing 3% bovine serum albumin and 0.1% Triton X-100 for 30 min. E-cadherin/actin was double-labeled by incubation with an anti-E-cadherin antibody (HECD-1 (1:200) or ECCD-2 (1:250); Zymed Laboratories) for 90 min, revealed by Cy2-labeled secondary antibody (Sigma) and by incubation with Alexa 546-labeled phalloidin (1:500) (Molecular Probes). HNF-4α was labeled by incubation with a polyclonal anti HNF-4α antibody (1:1000) (a gift from Dr. M. Pontoglio, CNRS Paris), revealed by Cy2-labeled secondary antibody (Sigma). The results were recorded with a Zeiss LSM510 confocal laser-scanning microscope equipped with a Zeiss Axiocam 100M oil immersion objective (plan Achromat 63 × 1.40 numerical aperture). The contrast and brightness settings were kept constant during the course of image acquisition.

Cells grown in suspension were incubated in ice-cold PBS, fixed in 4% paraformaldehyde in phosphate-buffered saline, and then centrifuged through Tek OCT (Shandon), and 8-μm cryosections were cut. Immunofluorescence proceedings were made as described above for attached cells. The results were recorded with a Zeiss Axiophot microscope equipped with oil immersion objectives (×100 Pan-Neofluor lenses) and analyzed with Axiovision software (release 4.3; Carl Zeiss).

Immunoblotting Analysis—Nuclear extracts were prepared as previously reported (35) with the presence in all buffers of protease inhibitors (protease inhibitor mixture; Sigma) and phosphatase inhibitors (2 mM Na₃VO₄, 50 mM NaF, 40 mM β-glycerophosphate). Protein concentrations were measured using the Bio-Rad DC protein assay (Bio-Rad) using bovine serum albumin as a standard. Samples (10 μg of nuclear proteins) were boiled for 5 min in SDS-reducing sample buffer and subjected to electrophoresis in reducing conditions in a 7.5% polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes (Bio-Rad) and probed successively with polyclonal anti HNF-4α (1:5000) and with anti-SP1 (1:500) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). A peroxidase-conjugated goat anti-rabbit IgG was used as secondary antibody (1:10000; Bio designs) and then analyzed with ECL Western blotting reagents (Amersham Biosciences). Red Pontceau staining was used to ensure that equal amounts of proteins were loaded.

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assays were performed as previously described (25). The AIV-E double-stranded oligonucleotide 5′-GGGAGAATGGTGGACTTTGCCCCCA-TGAGCCC-3′ was used as probe, as previously described (25). Supershift experiments were performed with antibody directed against HNF-4α (C-19X), according to the manufacturer’s instructions (Santa Cruz Biotechnology).

Reverse Transcription and Real Time PCR Analysis—Total RNA was isolated using RNAplus reagent (qBiogene) according to the manufacturer’s protocol. The reverse transcription (RT) experiments were performed as previously described (26) with 2 μg of total RNA in a total volume of 40 μl. Messenger RNA was quantified using the Light-Cycler system according to the manufacturer’s instructions (Roche Applied Science). PCRs were performed with a 1:50 final dilution of the RT samples. ApoA-IV mRNA level was quantified using hybridization probes designed by Genset (Proligo, Paris, France). PCR conditions were one step of denaturation (8 min at 95 °C) followed by 40 cycles (each cycle consisted of 15 s at 95 °C, 10 s at 58 °C, and 15 s at 72 °C). The mRNA levels of the other genes were quantified using SYBR Green, and the PCR conditions were one step of denaturation (8 min at 95 °C) followed by 40 cycles (each cycle consisted of 10 s at 95 °C, 10 s at 58–62 °C, depending of the cDNA amplified, and 10 s at 72 °C). The sequences of the primers used in this study were as follows: 5′-AGCCAGGACAAG-ACTCCTCTC-3′ (forward primer); 5′-TCTCACTCCACTGGA-C-3′ (reverse primer); 5′-CCCCCTTTGAGAGCTGCAGCTGGA-fluorescein isothiocyanate-3′ (anchor probe); and 5′-LCre640-CCCTGTTG-GCACTGCGCCC-3′-phosphate (detection probe) for ApoA-IV; 5′-T-TCAGCACCCTAGAGTCG-3′ (forward primer) and 5′-GTCTGA-GTCTCGAGGACAG-3′ (reverse primer) for microsomal transfer protein (MTP); 5′-GATCGGGAATGGCCGTTC-3′ (forward primer) and 5′-TGGAGATCTGACTGACTGTC-3′ (reverse primer) for DPP-IV; 5′-GGAGCAGGAGAGATGAGGTC-3′ (forward primer) and 5′-CCTTCCACACGTGATCCTT-3′ (reverse primer) for villin; 5′-AA-GATCGATGCGCCACTGATCA-3′ (forward primer) and 5′-TGGC- GTCTCGATTGTTTTA-3′ (reverse primer) for L19. The quantification of 18 S RNA (using Taqman probes from Applied Biosystems, Courtaboeuf, France) was used as control for the RNA extraction and reverse transcription experiments. Results were expressed as the ratio between the levels of mRNA of interest and 18 S RNA.

Statistical Analysis—Results were expressed as means ± S.E. or ± S.D. The statistical significance of differences was determined by an unpaired Student’s t test using Excel software.

RESULTS

Perturbation of Cell-Cell Adhesion Impairs ApoA-IV Gene Expression in Caco-2 Cells—The involvement of cell-cell adhesion in apoA-IV gene expression was first investigated either by a perturbation of E-cadherin adhesive properties or by disruption of the integrity of adherens junctions. For that means, high density-plated Caco-2 cells were first grown in normal calcium concentration until day 4 and then grown in the presence or in the absence of calcium for 48 h. As expected, calcium deprivation provoked cell rounding (Fig. 2A, a and b) and a loss of cell-cell adhesion. The disruption of adherens junctions was further characterized by confocal analysis. Calcium depletion led to a diffuse distribution of E-cadherin within some perinuclear aggregates (Fig. 2A, d); colocalization with the actin cytoskeleton was no longer observed (Fig. 2A, f). In contrast in control culture conditions E-cadherin staining was mostly concentrated at lateral membranes (Fig. 2A, c) and associated with the actin cytoskeleton staining (Fig. 2A, e). At the same time, calcium deprivation abolished the induction of apoA-IV mRNA expression that is observed between day 4 and day 6 in high density-plated cells grown in the presence of calcium (Fig. 2B). We next addressed whether E-cadherin perturbation affects other differentiation genes. Human intestinal dipeptidyl peptidase IV (DPP-IV) expression specifically increased during the enterocyctic differentiation (36). The level of DPP-IV mRNA increased between days 4 and 6, whereas calcium deprivation did not affect this increase (Fig. 2C). Similarly, the mRNA of villin, another differentiation gene (37), did not significantly vary upon calcium depletion (data not shown).

The crucial role of actin cytoskeleton integrity in the formation and maintenance of cell-cell adhesion is illustrated by the rapid and efficient disruption of cell-cell junctions caused by pharmacological agents that disrupt the actin cytoskeleton (e.g. cytochalasin D and Latrunculin B) (38–41). Therefore, Caco-2 cells, plated at low density, were grown until confluence (day 7) in normal calcium and then treated or not for
apoA-IV gene expression at the transcriptional level, we established a functional polarization of Caco-2 cells is improved as illustrated by the expression of the apoA-IV mRNA.

E-cadherin Controls ApoA-IV Gene Transcription

**FIGURE 2. Perturbation of cell-cell adhesion in Caco-2 cells by calcium switch impairs apoA-IV gene expression.** High density Caco-2 cells were cultured for 4 days with normal calcium concentration. At day 4, cells were cultured for 48 h with fresh serum-free medium containing (+) or not containing (−) calcium, to disrupt or not disrupt calcium-dependent cell-cell junctions. A, phase-contrast photographs of differentiating Caco-2 cells at day 6 in serum-free medium (a) and in serum- and calcium-free medium (b). Confocal microscopy images of E-cadherin (ECC2D antibody; green channel) and of F-actin network (phalloidin-Alexa 546; red channel) in the presence of calcium (c and e) or in calcium-free medium (d and f). c and d show E-cadherin staining, and e and f show a merge of E-cadherin and F-actin staining. Scale bar, 20 μm. B, quantification of the apoA-IV mRNA levels by RT real time PCR in differentiating Caco-2 cells at day 4 (D4) in calcium-containing medium and at day 6 (D6) in serum-free medium containing (+) or not (−) calcium. The data (apoA-IV/18S ratio) are means ± S.E. of three independent cultures performed in triplicate. ***, p ≤ 0.001 as determined by Student’s t test with respect to day 6 with calcium. C, quantification of the DPP-IV mRNA levels by RT real time PCR in Caco-2 cells treated as in B. The data (DPP-IV/18S ratio) are means ± S.E. of three independent cultures performed in triplicate.

48 h with 0.5 μM Latrunculin B (Fig. 3). As shown in Fig. 3A, cell morphology was greatly affected by Latrunculin B treatment. The loss of actin filaments (Fig. 3A, c and d) caused rounding of the cells (Fig. 3A, a and b) and affected the distribution of E-cadherin in lateral membranes. Indeed, E-cadherin staining was diffuse at the cell-cell contacts in Latrunculin B-treated cells, whereas we could observe a typical honeycomb E-cadherin staining strictly restricted to the cell-cell contact in control cells (Fig. 3A, e and f). Furthermore, no colocalization between E-cadherin and F-actin could be observed in treated cells (Fig. 3A, h), in contrast to control cells, where the merge yellow signal indicated that E-cadherin localized at adherens junctions was intimately associated with actin cytoskeleton (Fig. 3A, g). The addition of Latrunculin B completely blocked the induction of apoA-IV mRNA expression observed between days 7 and 9 in control cultures plated at low density (Fig. 3B). As for the calcium deprivation experiment, the addition of Latrunculin B did not modulate variations of the DPP-IV mRNA observed between days 7 and 9 (Fig. 3C).

Altogether, these results point out the importance of adherens junctions integrity, characterized by E-cadherin-actin anchorage, for the expression of the apoA-IV mRNA.

**ECM-cell Adhesion Controls Cell-Cell Adhesion and ApoA-IV Gene Transcription**—We have previously shown that culture of Caco-2 cells on ECM does not affect the E-cadherin level but reinforces its targeting to the lateral membrane at the cell-cell contacts. Consequently, the functional polarization of Caco-2 cells is improved as illustrated by the formation of large domes (30). In these conditions, apoA-IV mRNA level is increased (30). In order to determine whether ECM controls apoA-IV gene expression at the transcriptional level, we established a permanent Caco-2 cell line expressing the green fluorescent protein (GFP) under the control of the human apoA-IV promoter regulatory sequences responsible for the expression of apoA-IV in villus enterocytes in vivo (eC3A4-GFP cells) (Fig. 1) (24, 25). Fig. 4A shows that culture of the eC3A4-GFP cells on ECM boosted the expression of the reporter gene, as determined by measuring the intensity of the fluorescence by flow cytometry analysis. These eC3A4-GFP cells behaved like the parental Caco-2 cells; ECM reinforces the targeting of E-cadherin to lateral membranes (Fig. 4B) and induces the formation of large domes (Fig. 4C). The increased recruitment of E-cadherin at the lateral membrane is involved in the dome formation, since functional anti-E-cadherin antibodies (HECD1 and SHE78-7), which are known to block cell-cell adhesion (42), reduced the formation of domes by Caco-2 cells grown on ECM or on plastic at a high density (data not shown). Furthermore, domes exhibited an intense and homogenous GFP fluorescence, whereas cells grown on an inert support formed a flat poorly fluorescent monolayer (Fig. 4C).

The activation of the transcriptional activity of apoA-IV promoter by ECM was mediated by β1 integrin, as demonstrated by the impairment of this activity using the β1 integrin blocking antibody (6S6) (Fig. 4D). Accordingly, blocking β1 integrin function drastically impaired cortical actin organization and colocalization of E-cadherin and actin, as previously shown (30). Conversely, eC3A4-GFP cells cultured in the presence of a β1 integrin activating antibody (B3B11) exhibited an increase in GFP fluorescence (Fig. 4D).

These results demonstrate a β1 integrin-dependent effect of ECM, which combines the reinforcement of E-cadherin targeting to the lateral
E-cadherin Controls ApoA-IV Gene Transcription

We first used a specific anti-E-cadherin blocking antibody; after plating high-density eC3A4-Luc cells on plastic, with a poorly fluorescent pattern, whereas on ECM they formed domes that homogeneously displayed GFP fluorescence. Scale bar, 20 μm. D, analysis of apoA-IV promoter activity of eC3A4-GFP cells. The cells were plated out on an inert support in the presence or absence of activating anti-J1 integrin antibody (B3B11) or on ECM in the presence or not of functional blocking J1 integrin antibody (656). The data, expressed as the ratio of GFP induction compared with culture on an inert support, were calculated by computational analysis of confocal stack series.

We then addressed directly the role of E-cadherin-dependent cell-cell adhesion in the control of apoA-IV gene transcription, either by blocking or by activating E-cadherin in Caco-2 cells expressing the easily quantified reporter luciferase gene under the control of the apoA-IV regulatory sequences (eC3A4-Luc cells) (Fig. 1).

We used a specific anti-E-cadherin blocking antibody; after plating high-density cultures in normal calcium concentration, adherens junctions were briefly disrupted at day 4 by chelating extracellular calcium with 2 mM EGTA to allow antibody accessibility to E-cadherin. Junc
tions were subsequently allowed to re-establish by replacing the cells for 48 h in fresh medium containing (+/−) or not containing (+/+) calcium. During these 48 h, calcium-treated (+/+) cells were incubated with an anti-E-cadherin blocking antibody (SHE78-7) or with a mouse IgG as a control. The disruption of adherens junctions was further characterized by confocal analysis. IgG-treated cells exhibited an intense E-cadherin staining mostly restricted to the lateral membranes (Fig. 5A, a), where it colocalizes with the actin cytoskeleton (Fig. 5A, c and e, merge). Conversely, blocking the E-cadherin engagement with SHE78-7 during the calcium restoration led to a diffuse distribution of E-cadherin within some perinuclear aggregates (Fig. 5A, b) and to a loss of colocalization with the actin cytoskeleton (Fig. 5A, d and f, merge), as already observed when cells were grown in the absence of calcium (Fig. 2A, f). The specific blockade of E-cadherin engagement with the SHE78-7 antibody completely abolished the induction of apoA-IV transcriptional activity observed between day 4 and day 6 in control conditions, as did calcium deprivation from day 4 (Fig. 5B). This could not be imputed to the short EGTA treatment performed at day 4, which did not affect the apoA-IV induction (Fig. 5B). As expected from the results shown in Fig. 2C, the specific blockade of E-cadherin engagement did not alter the PPP-IV mRNA expression (Fig. 5C).

Altogether, these results indicate that the disruption of E-cadherin-dependent cell-cell adhesion impairs the activation of apoA-IV gene transcription.

The results described above are consistent with the fact that the integrity of E-cadherin-mediated cell-cell contact is necessary to the

FIGURE 4. ApoA-IV gene transcription and cell-cell junctions are induced by ECM. A, analysis of apoA-IV promoter activity by quantification of GFP expression by flow cytometry. eC3A4-GFP or mock-transfected cells were plated out at low density and cultured until confluence on plastic without ECM or on native ECM. The mean GFP fluorescence of the apoA-IV-GFP signal was measured by flow cytometry. Data are means ± S.D. of three independent experiments. **, differs from control without ECM at p < 0.01 determined by Student’s t test. B, eC3A4-GFP cells were plated out at low density on glass (a) or on native ECM (b), processed for immunofluorescence with anti-E-cadherin (ECCD2 antibody, green channel) at confluence, and analyzed by confocal microscopy. Note that E-cadherin is mostly located at the cell-cell junction membrane. Scale bar, 20 μm. C, eC3A4-GFP cells were plated out on plastic or on ECM, and GFP expression was analyzed by confocal microscopy at confluence. Z stack analysis was performed to visualize fluorescence along the whole height of domes. Note that the cells formed a flat monolayer on plastic, with a poorly fluorescent pattern, whereas on ECM they formed domes that homogeneously displayed GFP fluorescence. Scale bar, 50 μm. D, analysis of apoA-IV promoter activity of eC3A4-GFP cells. The cells were plated out on an inert support in the presence or absence of activating anti-J1 integrin antibody (B3B11) or on ECM in the presence or not of functional blocking J1 integrin antibody (656). The data, expressed as the ratio of GFP induction compared with culture on an inert support, were calculated by computational analysis of confocal stack series.

FIGURE 5. Perturbation of E-cadherin-dependent cell-cell adhesion in Caco-2 cells impairs apoA-IV gene transcription. High density eC3A4-Luc cells were cultured for 4 days with normal calcium concentration. At day 4, the cells were treated for 10 min with EGTA (2 mM) in order to open cell-cell junctions. The EGTA-containing medium was washed out and replaced for 48 h with fresh serum-free calcium-free medium (+/−) or serum-free calcium-containing medium (+/+) supplemented or not with IgG or E-cadherin functional blocking antibody SHE78-7 (10 μg/ml). A, confocal analysis of E-cadherin staining (ECCD2 antibody; green channel) and of the F-actin network (phalloidin-Alexa 546; red channel) in cells treated either with IgG (a, c, e, and g) or with E-cadherin blocking antibody (b, d, f, and h) and analyzed by confocal microscopy at confluence. Scale bar, 20 μm. B, eC3A4-Luc cells, cultured under the same conditions, were processed for luciferase activity. The data are means ± S.E. of three independent cultures performed in triplicate. §§§, p < 0.001 with respect to day 6 in normal calcium (+/+/); ***, p < 0.001 with respect to day 6 in normal calcium (+/+). Ca2+, calcium; Ca2+/H11001, confocal analysis of E-cadherin staining (ECCD2 antibody; green channel) and of the F-actin network (phalloidin-Alexa 546; red channel) in cells treated either with IgG (a, c, e, and g) or with E-cadherin blocking antibody (b, d, f, and h) and analyzed by confocal microscopy at confluence. Scale bar, 50 μm. C, eC3A4-GFP/Luc cells were cultured in triplicate. Ca2+, Ca2+; Ca2+/H11001, confocal analysis of E-cadherin staining (ECCD2 antibody; green channel) and of the F-actin network (phalloidin-Alexa 546; red channel) in cells treated either with IgG (a, c, e, and g) or with E-cadherin blocking antibody (b, d, f, and h) and analyzed by confocal microscopy at confluence. Scale bar, 50 μm.
E-cadherin Controls ApoA-IV Gene Transcription

induction of apoA-IV expression. The role of E-cadherin in the control of the apoA-IV gene expression was further investigated in fully differentiated cells in order to address the question of its role in the maintenance of expression of this differentiation gene. Cell-cell contacts were affected in Caco-2 cells after 16 days of culture (31) by deprivation of calcium for 2 days (Fig. 6A). As shown in Fig. 6B, apoA-IV gene transcrip-
tion was significantly decreased. Similarly, following Latrunculin B treatment that disrupts the actin cytoskeleton, apoA-IV gene transcription was decreased (Fig. 6B).

We then studied the effect of de novo formation of cell-cell junctions on the apoA-IV promoter activity. eC3A4-Luc cells were grown in the absence of calcium to prevent the formation of intercellular junctions and then switched or not to calcium-containing medium at day 4 to induce cell-cell adhesion. Phase microscopy analysis showed that the addition of calcium-containing medium (+/+) allowed cells to adopt an epithelial morphology (Fig. 7A, b) as opposed to the rounded cells cultured in the absence of calcium (−/−) (Fig. 7A, a). Accordingly, luciferase activity was increased in cells switched to calcium-containing medium, indicating that activation of apoA-IV gene regulatory sequences paralleled the onset of cell-cell adhesion (Fig. 7B). Forcing cell aggregation in suspension can also induce cell-cell junctions and rules out the contribution of cell-ECM adhesion. As soon as after 2 days of culture, suspended eC3A4-Luc cells formed small compact aggregates. E-cadherin displayed a punctuate staining all along the cell-cell contacts (Fig. 7C, a). After 6 days, aggregates increased in size, and E-cadherin staining was markedly reinforced and was accompanied by the formation of a strong cortical actin network (Fig. 7C, b and d). The aggregation of eC3A4-Luc cells in suspension culture was sufficient to induce the transcriptional activation of the apoA-IV promoter, with kinetics similar to adherent cells (Fig. 7D). This reinforces the hypothesis that E-cadherin-mediated cell-cell adhesion is able to trigger signals controlling apoA-IV transcription.

To ascertain a role for the E-cadherin engagement in the transcriptional activation of the apoA-IV promoter, we used the E-cadherin recombinant chimera Ecad-Fc, in which the extracellular domain of E-cadherin is fused to the Fc fragment of immunoglobulin. N- or E-cadherin recombinant chimeras have recently been developed as tools to control cadherin homophilic binding and to study cellular responses triggered by cadherin activation (3, 43, 44). Selective engagement of E-cadherin was achieved by plating eC3A4-Luc cells on Ecad-Fc-coated surfaces at low density, in order to limit the formation of cell-cell contacts (Fig. 8A, b). Control cells were plated on anti-Fc-antibody-coated surface (Fig. 8A, a). Phase microscopy images showed that cells spread on E-cad-Fc and displayed an elongated shape, whereas they formed typical islands containing small cells on anti-Fc antibody (Fig. 8A, see the enlargement in the bottom insets). ApoA-IV promoter transcriptional activity was assessed as previously by measurement of luciferase activity (Fig. 8B). We observed a 2.5-fold activation in cells grown on E-cad-Fc, as compared with cells grown on anti-Fc antibody. Consistently, the mRNA levels of apoA-IV were increased by 2.7 in these conditions (Fig. 8C). By contrast, the mRNA levels of villin remained
E-cadherin Controls ApoA-IV Gene Transcription

We then investigated by electrophoretic mobility shift assay experiments whether the DNA binding activity of HNF-4α was affected by E-cadherin perturbation. HNF-4α binds two HRE located in the apoA-IV promoter region, designated AIV-C (−122 to −148) and AIV-E (−357 to −377) (25, 27), which bind HNF-4α in nuclear extracts from Caco-2 cells (26) (Fig. 9C, lanes 1 and 2). As shown in Fig. 9C, HNF-4α binding to AIV-E was significantly decreased by blocking the reformation of the junctions after a short EGTA treatment, either by using calcium-free medium (Fig. 9C, lane 6) or by specifically blocking E-cadherin engagement with the SHE78-7 antibody (Fig. 9C, lane 4).

We then determined whether the transcriptional effect of E-cadherin-dependent cell-cell adhesion extended to another intestine-specific gene previously shown to be controlled by HNF-4α, namely the MTP (46). As observed in Fig. 9D, MTP mRNA levels were significantly decreased in calcium-free conditions. As shown in Figs. 2C and 5C, DPP-IV mRNA levels (as well as villin mRNA levels; data not shown)

unchanged, and the mRNA levels of DPP-IV exhibited only a slight increase (Fig. 8C). This result demonstrates that a direct E-cadherin activation is involved in the transcriptional activation of the apoA-IV promoter.

E-cadherin-dependent Adhesion Controls Nuclear HNF-4α Amount and Activity—The effect of E-cadherin perturbation on apoA-IV transcription prompted us to investigate whether the disruption of E-cadherin-dependent cell-cell adhesion also affects the transcription factor HNF-4α, which we recently demonstrated controlled the enterocyte-specific expression of apoA-IV in vivo (25, 27) and in Caco-2 cells (25, 27). We determined the level of HNF-4α by Western blot in nuclear extracts from cells cultured in our different experimental culture conditions. We observed a significant decrease of HNF-4α amount in nuclear extracts from cells either treated by E-cadherin blocking antibody or submitted to calcium depletion, two conditions causing disruption of E-cadherin-dependent cell-cell adhesion (Fig. 9A, top). The specificity of this effect was assessed by the observation that the level of the transcription factor SP1, also described to control apoA-IV gene transcription (45), was not affected in these conditions (Fig. 9A, bottom). The decrease of HNF-4α cell content in these experimental conditions was further confirmed by immunofluorescence detection of a lower signal in cells treated with the E-cadherin blocking antibody (Fig. 9B).

FIGURE 8. Direct activation of E-cadherin induces apoA-IV gene transcription. A, phase-contrast photographs of eC3A4-Luc cells plated out at low density and grown for 48 h on recombinant human E-cadherin-Fc chimera-coated plate (E-cad-Fc) (b) or on inert support precoated with anti-Fc IgG (control) (a) (see also enlargements in the insets). Scale bars, 20 μm. B, eC3A4-Luc cells, cultured under the same conditions as in A, were processed for luciferase activity. The data are means ± S.E. of two independent cultures performed in triplicate ***, p < 0.001 as determined by Student’s t test with respect to the control cells. C, quantification of the apoA-IV, DPP-IV, and villin mRNA in eC3A4-Luc cells cultured as described above on an E-cadherin-Fc chimera-coated plate (+) or on inert support precoated with anti-Fc IgG (−). The data are means ± S.E. of two independent cultures performed in triplicate, p < 0.05 as determined by Student’s t test with respect to the control cells.
were not affected upon E-cadherin-dependent cell adhesion perturbation. These genes are induced during Caco-2 cell differentiation but not through HNF-4α. As expected, the mRNA levels of the large ribosomal polypeptide L19, used as an internal control, were not affected in this condition (Fig. 9D).

Altogether, these results strongly suggest the involvement of HNF-4α in the E-cadherin-dependent transcriptional control of intestine-specific genes such as the apoA-IV gene.

**DISCUSSION**

Cell adhesion mediated by cadherins or integrins has been shown to play important roles throughout the differentiation process of many tissues (1, 47–51). In the present study, we show that intact E-cadherin-dependent junctions are required for the transcriptional activation of a specific marker of enterocyte differentiation, the apoA-IV gene. Furthermore, we demonstrate for the first time that a direct activation of E-cadherin is sufficient to activate the transcription of an intestinal gene. This effect is associated with a modulation of the amount and binding activity of the transcription factor HNF-4α.

We showed that inhibition of the adhesive function of E-cadherin by calcium depletion lowered apoA-IV expression, as did the disruption of actin filaments. A controlled actin polymerization is essential for the stabilization of E-cadherin-mediated adhesion. Cell-cell adhesion is strengthened by clustering of cadherin receptors at junctions and by their association with the actin cytoskeleton (16, 39). As we previously reported, the organization of the apical actin cytoskeleton and the formation of functional E-cadherin-actin complexes are induced by ECM in enterocytes (30). Here, we displayed a parallel induction of apoA-IV promoter activity by ECM, which was impaired by the anti-β1 integrin blocking antibody and was mimicked by the anti-β1 integrin activating antibody in cells grown on an inert support. Altogether, these data raised the question of a control of the transcriptional activity of apoA-IV gene by functional adherens junctions.

A role for E-cadherin in intestinal differentiation was first supported by works from Hermiston and Gordon (4), who examined the contribution of E-cadherin to intestinal homeostasis in vivo. Expression of a dominant negative N/E-cadherin mutant in villous enterocytes resulted in the perturbation of cell-cell adhesion, associated with an increased enterocyte migration rate along the crypt to villus axis, a loss of the differentiated polarized phenotype, and increased apoptosis (4). Interestingly, E-cadherin expression is weaker in proliferative cells located at the bottom of the human intestinal crypt (15). The importance of E-cadherin-dependent junctions in the acquisition and the maintenance of the enterocytic phenotype (i.e. expression of intestinal genes and/or acquisition of differentiated morphologic features) has also been strongly suggested by cell culture studies manipulating the cell density or using calcium switch methods (52, 53). Such a role has first been evidenced in muscular differentiation by functional inhibition of N-cadherin-mediated contact (47, 48, 54–56). Consistently, we show that E-cadherin perturbation using calcium depletion or actin filament disruption impairs apoA-IV transcription at the time of differentiation onset as well as once the differentiation has been fully established. Our results on the impairment of apoA-IV gene expression by E-cadherin blocking antibodies definitively demonstrate the role of the E-cadherin-dependent junction in this process. If cell-cell contacts are necessary for the differentiation of Caco-2 cells (23, 30, 31), it is noteworthy that impairing E-cadherin-mediated junctions does not result in a general block of differentiation. Indeed, apoA-IV and MTP gene expression are down-regulated upon E-cadherin perturbation, whereas DPP-IV and villin genes, which are other differentiation markers, are not affected.

**E-cadherin Controls ApoA-IV Gene Transcription**

The cross-talk between cell-cell and cell-matrix adhesion pathways, as well as the secondary signals transmitted by polarization induced by E-cadherin activation, make it difficult to obtain evidence for a direct role of E-cadherin activation in cell differentiation. However, the aggregation assay suggests that the control of apoA-IV gene expression by E-cadherin is not dependent on cell-ECM adhesion. Nevertheless, the contribution of other adhesion receptors, as a relay of the signal from the E-cadherin activation, cannot be excluded. Using the cad-Fc recombinant chimera, a powerful tool developed to decipher cadherin-initiated signaling pathways (3, 43, 57–59), an *in vitro* study has recently shown that N-cadherin activation was able to trigger myogenic cell differentiation autonomously (i.e. without the contribution of any other adhesion molecule) (3). Our data with chimeric E-cadherin suggest that activation of the E-cadherin signaling pathway is sufficient to induce apoA-IV gene transcription. This demonstrates the role of direct E-cadherin engagement in intestinal differentiation, independent of the cell density.

Finally, our results demonstrate directly for the first time a role for E-cadherin in the control of the nuclear abundance and the binding activity of a transcription factor involved in cell differentiation. Indeed, we showed here that the amount and binding activity of nuclear HNF-4α depended on the integrity of E-cadherin-dependent junctions. Precisely, we recently demonstrated that apoA-IV gene expression was specifically controlled by HNF-4α in intestinal epithelial cells *in vivo* (27) and in response to dietary lipids (26) via the same DNA regulatory region as that involved in E-cadherin-mediated activation. Altogether, these results strongly suggest that E-cadherin engagement controls the apoA-IV gene expression through the control of HNF-4α nuclear abundance, by a mechanism which remains to be elucidated. The activity of HNF-4α has been shown to depend on specific protein-protein interactions (60, 61), on acetylation (62), or on phosphorylation (63–67), but the kinases involved are still debated. However, few studies correlate the modulation of HNF-4α activity with HNF-4α protein level (66, 67).

HNF-4α is known to play a pivotal role in epithelial differentiation, regulating the expression of genes involved in nutrient metabolism and transport (for a review, see Ref. 68). Its invalidation in the adult liver provokes lethal defects (69, 70). It has also been shown to be involved in the onset of intestinal development *in vivo* in *Drosophila*, in which the intestinal developmental stops in the absence of the HNF-4α homolog (71). Moreover, HNF-4α is expressed in the primitive endoderm as early as it becomes morphologically distinct (72, 73). Liver-specific deletion of HNF-4α in the embryo (74) led to the disorganization of liver architecture with an absence of normal cell-cell contacts and membrane E-cadherin and ZO1 staining in mutant hepatocytes (75). Conversely, forced expression of HNF-4α in cultured hepatoma cells resulted, besides the expected activation of liver-specific gene expression, in the establishment of an appropriate cellular morphology, including epithelial cell adhesion (76), through the induction of the expression of E-cadherin and of the intermediate filament cytokeratin proteins (77). Thus, the morphogenic potential of HNF-4α acts in hepatic cells via the activation of the E-cadherin gene.

From our own results and these observations, it is tempting to put HNF-4α at the crossroad between cell-cell adhesion and the expression of differentiated functions in epithelial cells. HNF-4α controls morphological differentiation through the expression of E-cadherin gene and the subsequent E-cadherin-mediated cell-cell adhesion, which, in turn, controls nuclear accumulation of HNF-4α and the subsequent activation of genes responsible for differentiated functions. Similarly, the caudal-related Cdx transcription factors (Cdx1 and Cdx2) have also been reported to regulate intestine-specific gene expression and to induce E-cadherin activity and cell-cell adhesion (78). Moreover, E-cadherin-
mediated cell-cell contact triggers p38 mitogen-activated protein kinase cascade activation (53), which has been shown to enhance the transactivation capacity of CdX2 (52). This calls into question the specific contribution and possible cooperation of HNF-4α and CdX factors in the control of epithelium function through cell-cell adhesion.

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