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E-cadherin regulates MAL-SRF-mediated transcription in epithelial cells

Stephan Busche[1], Elisabeth Kremmer[2] and Guido Posern[1,*]

[1]Department of Molecular Biology, AG Regulation of Gene Expression, Max Planck Institute of Biochemistry, D-82152 Martinsried, Germany
[2]Helmholtz Zentrum München, Institute of Molecular Immunology, D-81377 Munich, Germany
*Author for correspondence (posern@biochem.mpg.de)

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Summary

Epithelial junctions are dynamically and functionally linked to the actin cytoskeleton, and their disassembly is a key event during physiological and pathological processes. We recently showed that epithelial disintegration facilitates transcriptional activation via Rac, G-actin, MAL (also known as MRTF) and serum response factor (SRF). Here, we investigate which specific component of the epithelial junction is essential for this MAL-SRF-mediated transcription. The Ca^{2+}-dependent dissociation of polarised epithelial cells depleted of ZO proteins – which form adherens junctions (AJs) but completely lack tight junctions (TJs) – fully activated SRF. By contrast, AGS gastric adenocarcinoma epithelial cells, which form TJs but are deficient in E-cadherin, and therefore also in AJs, failed to activate SRF. The introduction of wild-type E-cadherin in AGS cells restored AJ formation and MAL-SRF inducibility. To gain further insight into the membrane-proximal signalling, AGS cells were stably transfected with E-cadherin–α-catenin fusions. Despite restored formation of cell-cell contacts containing the nectin-afadin complex and p120-catenin, these cells did not activate SRF upon junction dissociation, suggesting that signal transmission depends on the C-terminal tail of E-cadherin. We conclude that the dissociation of intercellular E-cadherin interactions from AJs, and signals originating from the C-terminal region covering the β-catenin-binding site of E-cadherin, are essential for transcriptional activation via Rac, MAL and SRF, whereas TJs are not involved.

Key words: Adherens junctions, Tight junctions, Gene expression

Introduction

Epithelial cells are laterally connected via adhesive structures, including tight junctions (TJs) and adherens junctions (AJs) (Farquhar and Palade, 1963). Located most apically, the TJs are locally clustered in TJ plaque structures via their cytoplasmic scaffolding partners, the zonula occludens proteins ZO-1, ZO-2 and ZO-3 (Itoh et al., 1999). These associate via their C-terminus with actin and thereby provide a direct link to the cytoskeleton. Epithelial cells depleted for all three ZO-protein family members were shown to lack TJs but still polarise and form fully developed AJs (Umeda et al., 2006). AJs, which are located immediately basal to the TJs, are involved in the initiation and stabilisation of cell-cell adhesion, the regulation of the actin cytoskeleton, and intracellular signalling (Gumbiner, 2005). Their formation is mediated by the Ca^{2+}-dependent homophilic *trans*-interaction of classical cadherins (Gumbiner et al., 1988). The 70 distal amino acids of the cytoplasmic domain are essential for β-catenin binding (Nagafuchi et al., 1994), which in turn binds to α-catenin and mediates a dynamic interaction with the actin cytoskeleton (Drees et al., 2005; Yamada et al., 2005). A fusion protein of E-cadherin lacking the β-catenin-binding domain and the adhesion modulation domain of α-catenin is sufficient for the formation of cadherin-based cell adhesion (Imamura et al., 1999).

The integrity of epithelial cell-cell contacts is implicated in transcriptional regulation, e.g. during junctional disintegration and epithelial-mesenchymal transition (EMT) (Gottardi et al., 2001; Morita et al., 2007). We and others have recently shown that dissociation of epithelial cell-cell contacts, either by reduction of extracellular Ca^{2+} or by inducing EMT and cell scattering, initiates transcription via the transcription factor serum response factor (SRF) (Fan et al., 2007; Morita et al., 2007; Busche et al., 2008). Thereby, the coactivator MAL (also known as MRTF and MKL) is essential for the activation of a subset of SRF target genes, including those encoding vinculin (*Vcl*) and smooth muscle α-actin (*Acta2*). Within the signalling cascade, we identified Rac and monomeric actin, which forms a repressive complex with MAL, as essential and sufficient for signal transmission (Busche et al., 2008). However, the precise junctional component triggering the MAL-SRF pathway and subsequent transcription remained elusive.

Here, we show that disintegrating AJs rather than TJs are the essential junctional components that induce signalling towards MAL-SRF. The mouse mammary epithelial cell line EpH4 as well as TJ-deficient derivatives activated an SRF reporter and known endogenous target genes upon junction dissociation. Conversely, reintroduction of E-cadherin into an AJ-deficient cell line restored SRF inducibility. AJ-deficient cells stably transfected with mutant E-cadherin–α-catenin fusions restored the formation of E-cadherin-containing cell contacts that included the nectin-afadin complex, but did not activate SRF upon junction dissociation. Thereby, the formation of epithelial junctions and SRF induction were uncoupled by mutant E-cadherins lacking their C-terminal domain. This suggests that MAL-SRF regulation in epithelial cells occurs through E-cadherin and requires the intracellular region containing the β-catenin-binding domain.

Results

Epithelial junction dissociation activates SRF independent of tight junctions

Epithelial cell-cell contacts can regulate transcription and gene expression. We previously showed that the dissociation of epithelial

junctions induces MAL-SRF-dependent target gene expression via Rac and actin signalling (Busche et al., 2008). However, it remained unclear which components are essential for signalling to SRF, because the withdrawal of extracellular Ca^{2+} disrupts the entire epithelial junctional complex, including AJs and TJs (Martinez-Palomo et al., 1980; Kartenbeck et al., 1991; Umeda et al., 2004; Busche et al., 2008; Yamazaki et al., 2008). To directly test the involvement of TJs, we used mouse mammary epithelial EpH4 cells specifically depleted for crucial TJ components (Umeda et al., 2006). Both *ZO-1*$^{-/-}$ and *ZO-1*$^{-/-}$/*ZO-2*^{kd} cell lines showed no ZO-1 membrane localisation (Fig. 1A). In the TJ-deficient *ZO-1*$^{-/-}$/*ZO-2*^{kd} cell line, the efficiency of ZO-2 knockdown was determined by quantitative real-time reverse transcriptase (RT)-PCR to be around 85% (Fig. 1B).

Despite their different TJ composition, all three cell lines polarised well and formed fully developed AJs, confirmed by membrane-bound E-cadherin; these junctions were readily dissociated upon Ca^{2+} switch to 0.02 mM (Umeda et al., 2006) (Fig. 1A). Transfection with a MAL-SRF-regulated luciferase reporter revealed a significant induction upon Ca^{2+} switch in all three confluently grown cell lines (Fig. 1C), strongly suggesting that TJs are dispensable for SRF activation. Next we analysed the known endogenous MAL-SRF target gene *Vcl* (Gineitis and Treisman, 2001) via quantitative real-time RT-PCR. At 3 hours after Ca^{2+} switch, *Vcl* was upregulated in all three cell lines independently of TJ existence (Fig. 1D). Its basal expression level was independent of TJs, and it was induced around three- to fourfold (Fig. 1D). Similar results were obtained for another known MAL-SRF target, *Acta2* (not shown) (Du et al., 2004).

In contrast to *Vcl*, the basal activity of the SRF reporter was slightly elevated in *ZO-1*$^{-/-}$ and *ZO-1*$^{-/-}$/*ZO-2*^{kd} cells. This could potentially indicate that TJs have a suppressive effect on SRF, possibly through TJ-mediated stabilisation of the cortical actin cytoskeleton, the dynamics of which are required for MAL-regulated SRF activity. However, staining of the F-actin cytoskeleton in control and knockdown cells did not reveal any obvious changes in the organisation of cortical actin (Fig. 1E). Moreover, we tested whether the absence of TJs restores SRF induction by serum, because we recently showed that epithelial cells are barely serum stimulatable (Busche et al., 2008). Again, this was not altered; all three cell lines failed to exhibit considerable reporter activation by serum, suggesting that ZO-1 and ZO-2 do not fundamentally affect serum signalling through actin (data not shown). Together, the results strongly suggest that TJs are not required for MAL-SRF reporter activation and target gene expression upon dissociation of epithelial junctions. TJs can thus

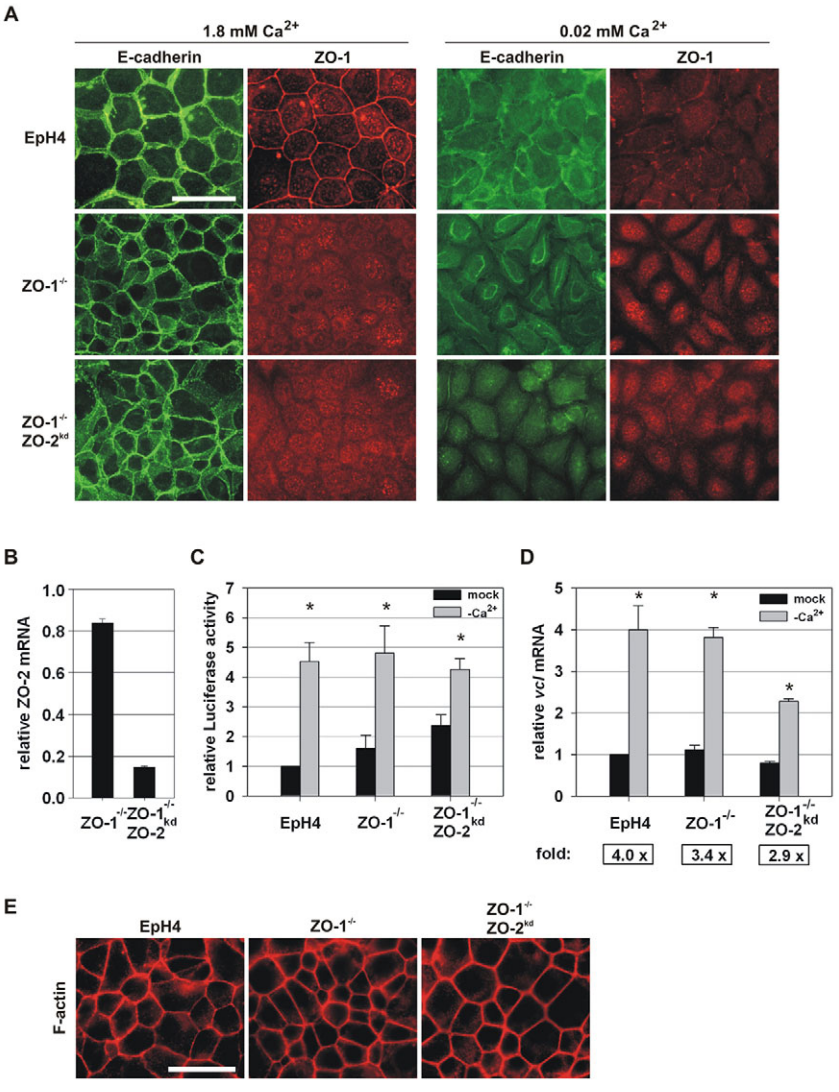

Fig. 1. MAL-SRF activation by epithelial junction dissociation does not depend on TJs. (**A**) Junctional characterisation of EpH4, *ZO-1*$^{-/-}$ and *ZO-1*$^{-/-}$/*ZO-2*^{kd} cells. Cells were grown to confluency for 48 hours. At 7 hours after the medium exchange to the indicated Ca^{2+} concentrations, the cells were fixed and immunostained for E-cadherin (clone 36) and ZO-1. (**B**) Endogenous *ZO-2* mRNA expression level in *ZO-1*$^{-/-}$ and *ZO-1*$^{-/-}$/*ZO-2*^{kd} cells, relative to the parental EpH4. Total RNA was isolated and analysed by quantitative RT-PCR. (**C**) EpH4, *ZO-1*$^{-/-}$ and *ZO-1*$^{-/-}$/*ZO-2*^{kd} cells were transiently transfected with a MAL-SRF reporter and subsequently seeded to form a confluent monolayer. 24 hours later the Ca^{2+} switch was performed and luciferase activity was measured 7 hours later. Shown is the relative luciferase activity normalised to pRL-TK. (**D**) Induction of the endogenous SRF target gene *Vcl* upon junction dissociation. Shown is the relative mRNA induction normalised to mock-treated EpH4 cells. Numbers below indicate the fold induction within each cell line. (**E**) F-actin staining (phalloidin) of EpH4, *ZO-1*$^{-/-}$ and *ZO-1*$^{-/-}$/*ZO-2*^{kd} cells grown as in A. Mock, medium exchange with 1.8 mM Ca^{2+}; $-Ca^{2+}$, medium exchange with 0.02 mM Ca^{2+}. Error bars represent s.e.m. of at least three independent experiments. *Statistical significance at $P<0.05$ according to unpaired Student's *t*-test. Scale bars: 50 μm.

be largely excluded as the molecular sensor of epithelial integrity for MAL-SRF-dependent transcription.

E-cadherin is sufficient for MAL-SRF induction triggered by AJ dissociation

We next investigated whether E-cadherin-containing AJs are sufficient for MAL-SRF activation upon epithelial junction dissociation. The AGS gastric adenocarcinoma and MDA-MB 435S melanoma cell lines are E-cadherin and therefore AJ deficient, and both do not activate SRF upon Ca^{2+} withdrawal despite a functional downstream pathway (Busche et al., 2008). We thus restored E-cadherin expression in AGS cells by stably transfecting them with wild-type E-cadherin. Two distinct monoclonal AGS cell lines stably expressing E-cadherin formed fully developed epithelial sheets that contained proper TJs and AJs (Fig. 2 and supplementary material Fig. S1). Detailed characterisation by immunofluorescence revealed that the AJ components β-catenin, p120-catenin, afadin and nectin clearly localised at the cell-cell contacts of E-cadherin-expressing cells only (Fig. 2). Moreover, cortical F-actin staining was also restored in normal medium, but this localised staining, together with that of the junctional components, was readily lost when cells were exposed to conditions of low Ca^{2+}.

By contrast, control cells grown in a confluent monolayer formed partially developed epithelial sheets, visualised by only partial obliteration of cell-cell borders in phase-contrast microscopy (Fig. 2A). Although they lacked localisation of β-catenin, p120-catenin, nectin and afadin, ZO-1 localised inhomogeneously at cell-cell junctions. Upon Ca^{2+} withdrawal, the partially interconnected cells dissociated completely, and ZO-1 dislocated from the membrane (Fig. 2A). We speculate that these effects were due to disassembly of Ca^{2+}-dependent desmosomal cadherins.

We then looked at whether SRF inducibility was restored. In E-cadherin re-expressing cells, Ca^{2+} withdrawal induced a significant SRF-reporter activation, accompanied by epithelial-sheet dissociation (Fig. 3A). Also, expression of known endogenous MAL-SRF target genes such as *Vcl* was significantly upregulated (Fig. 3B). In both cases, E-cadherin expression correlated with the induction level (Fig. 3A, inset). Moreover, the fold induction in low Ca^{2+} conditions was comparable to normal epithelial cells (Fig. 1) (Busche et al., 2008). By contrast, SRF and known target genes were not activated in control cells during dissociation and ZO-1 dislocation, consistent with the dispensability of TJs and desmosomes for SRF activation (Fig. 3A,B). Overall, our data demonstrate that the E-cadherin-containing AJs are sufficient and required for SRF activation upon epithelial disintegration.

In some cell types, activation of MAL correlates with its translocation from the cytoplasm to the nucleus. Hence, the localisation of MAL in the AGS cell lines with and without restored E-cadherin expression was analysed. In the control cells, MAL was constitutively enriched in the nuclear region, although there was no transcriptional activation (Fig. 3C). This suggests that the activity of MAL is inhibited even if the protein is permanently localised in the nucleus, consistent with previous observations (Vartiainen et al., 2007). By contrast, MAL staining in cells with restored E-cadherin expression was evenly visible throughout the cell body under normal conditions, whereas Ca^{2+} withdrawal induced a rapid relocalisation that correlated with the induction of SRF and with an increased level of *Vcl* mRNA.

We previously showed that the small GTPase Rac plays an important role in MAL-SRF activation in dissociating epithelial cells (Busche et al., 2008). Performing pull-down experiments for GTP-loaded Rac showed that Rac activity is not affected by low Ca^{2+} in control AGS cells, which lack E-cadherin (Fig. 3D). By contrast, cells with restored E-cadherin expression exhibited a considerably increased GTP loading of Rac following Ca^{2+} withdrawal for 10 minutes (Fig. 3D, right panels). The basal level of GTP-loaded Rac seemed to be slightly reduced in the unstimulated E-cadherin-expressing cells, consistent with the observed MAL relocation and the marginally lowered basal SRF activity. Together, the result suggests that the correlation between Rac activation and MAL-SRF-mediated transcription depends on E-cadherin expression.

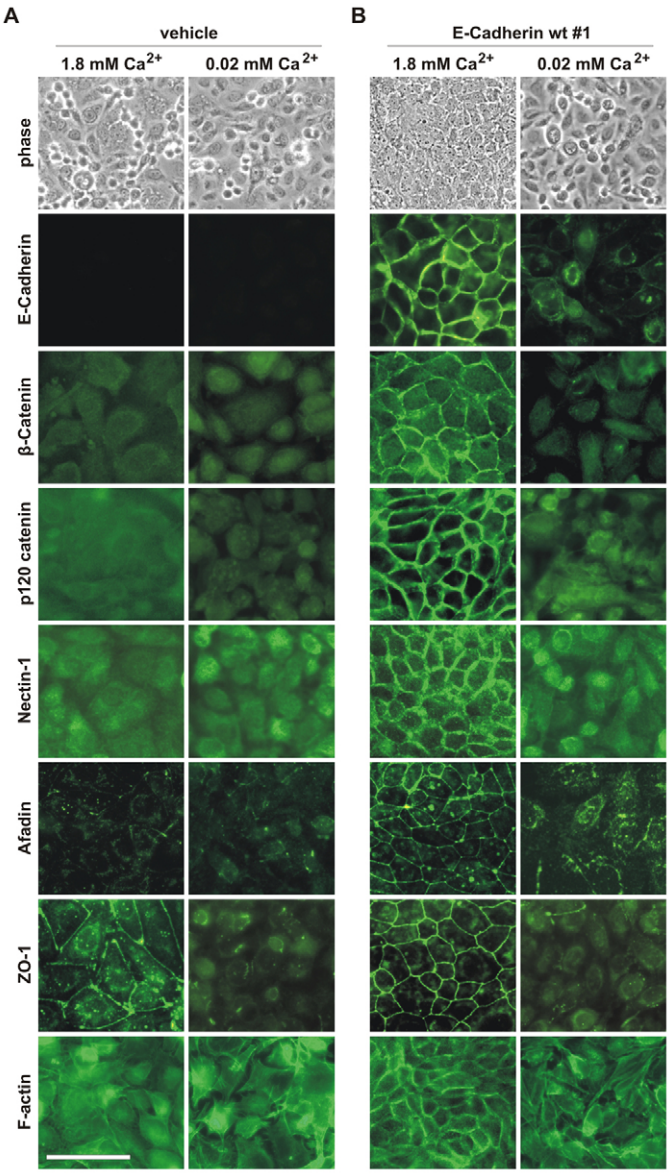

Fig. 2. Junctional characterisation of AGS cells stably expressing wild-type E-cadherin. Immunofluorescence micrographs of cells stably transfected with (**A**) empty vector (vehicle) or (**B**) wild-type E-cadherin (wt #1), grown to confluency for 48 hours. Cells were fixed 7 hours after Ca^{2+} switch and stained for E-cadherin (ECCD-2), β-catenin, p120-catenin, nectin-1, afadin, ZO-1 and F-actin (phalloidin). The top panels show phase-contrast images to visualise epithelial-sheet integrity.

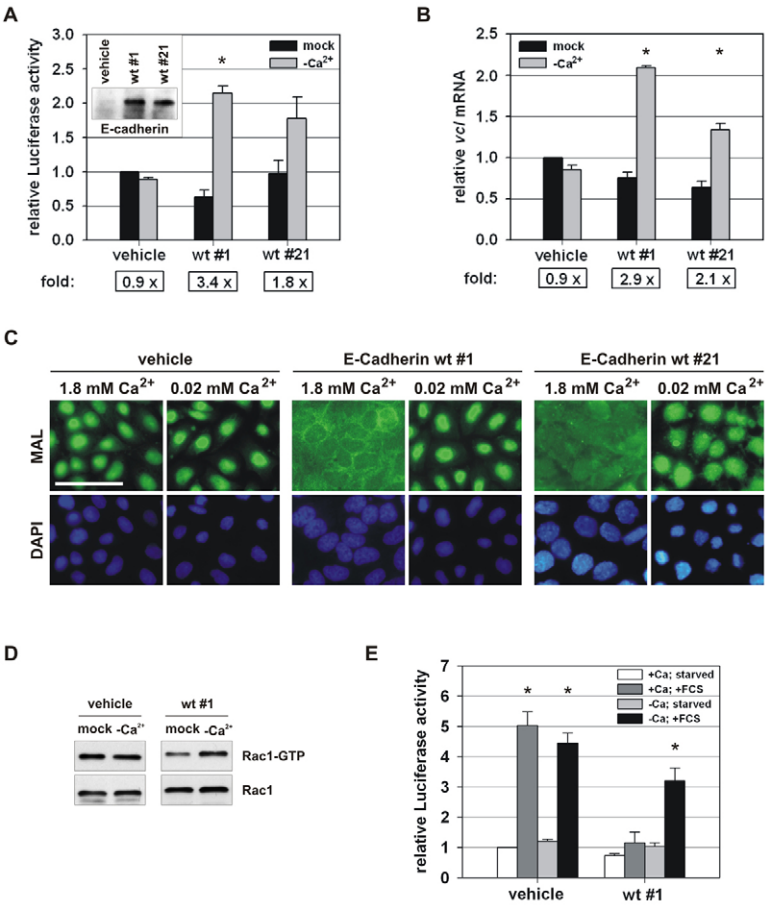

Fig. 3. Rescue of MAL-SRF activation by restored E-cadherin expression. (**A**) E-cadherin expression in AGS cells reconstitutes SRF inducibility by epithelial dissociation in low Ca^{2+}. Shown is the relative luciferase activity normalised to pRL-TK for two independently selected E-cadherin-expressing clones (wt #1 and wt #21). Numbers below indicate the fold induction within each cell line. Inset: E-cadherin expression levels, determined by western blotting using anti-E-cadherin antibody (clone 36). (**B**) Induction of the endogenous SRF target gene *Vcl* upon junction dissociation. Shown is the relative mRNA induction normalised to mock-treated vehicle AGS cells. (**C**) MAL localisation. Cells were grown as in Fig. 2, fixed 30 minutes after Ca^{2+} switch, and stained for MAL and DNA (DAPI). (**D**) Rac activation upon Ca^{2+} withdrawal from cells with restored E-cadherin expression (right panels). GTP-loaded Rac was precipitated from cell lysates after exchange to either normal- or low-Ca^{2+} medium for 10 minutes, followed by immunoblotting. Left panels: vehicle-transfected control cells for comparison. Total Rac is shown below as controls. (**E**) Intact E-cadherin-mediated cell contacts repress serum inducibility of MAL-SRF. Confluent AGS cells in normal Ca^{2+} medium (+Ca) were serum starved (0.2% FCS) for 24 hours (starved) followed by a Ca^{2+} switch (–Ca) and/or serum stimulus (15% FCS; +FCS) for 7 hours. Mock, medium exchange with 1.8 mM Ca^{2+}; $–Ca^{2+}$, medium exchange with 0.02 mM Ca^{2+}. Error bars indicate s.e.m. (n=3). *Statistical significance at $P<0.05$ according to unpaired Student's t-test. Scale bar: 50 μm.

Prompted by the finding that restored AJs result in MAL relocalisation, it was investigated whether E-cadherin expression also affects serum signalling to MAL-SRF. Cells were serum starved for 24 hours and subsequently serum stimulated for 7 hours in the presence or absence of Ca^{2+}. Interestingly, under these conditions cells exhibited a considerable serum induction of the reporter only when E-cadherin was either not expressed or dissociated by low Ca^{2+} (Fig. 3E). This result suggests a dual function of E-cadherin in epithelial cells: engaged E-cadherin inhibits serum signalling, resulting in loss of ability to be stimulated by serum, whereas dissociating E-cadherin activates MAL-SRF signalling (see Discussion).

The C-terminal region of E-cadherin is required for MAL-SRF induction

Following the identification of E-cadherin as the crucial sensor for dissociation-induced MAL-SRF activity, we wanted to investigate which domains of E-cadherin, and its interaction partners, are required for intracellular signalling. The cytoplasmic tail of E-cadherin binds to β-catenin, which in turn establishes a dynamic connection to the actin cytoskeleton through α-catenin (and probably other interaction partners) (Drees et al., 2005; Yamada et al., 2005). We therefore generated E-cadherin–α-catenin fusion proteins, which were shown to mediate cadherin-dependent cell-cell contacts in E-cadherin-deficient cells (Nagafuchi et al., 1994).

The proximal part of the fusions consisted of truncated E-cadherin lacking the C-terminal 73 amino acids, including the β-catenin-binding domain. This was fused to either the adhesion modulation domain of α-catenin (EΔβ–αAMD) (Imamura et al., 1999), the C-terminal part of α-catenin (EΔβ–αCT) or full-length α-catenin (EΔβ–αFL) (Fig. 4A). The fusion proteins were stably transfected in AGS cells, and three independent clones of each were analysed, showing expression of the fusions at the expected molecular weight (Fig. 4B). Even the shortest construct, EΔβ–αAMD, showed by confocal microscopy a clear localisation of the expressed fusion to epithelial junctions (Fig. 4C). Moreover, these junctions readily dissociated in low Ca^{2+} medium, and the staining was essentially indistinguishable from wild-type E-cadherin.

Strikingly, in contrast to wild-type E-cadherin, none of the E-cadherin fusions restored SRF activation upon Ca^{2+} withdrawal (Fig. 4D). Consistently, a relocalisation of MAL could not be observed, and Rac GTP loading did not increase (supplementary material Fig. S2). Yet, formation and dissociation of AJs was comparable in all instances. Even the longest fusion construct, containing the entire α-catenin open reading frame, failed to show any regulation of MAL-SRF-mediated transcription. This surprising finding largely excluded the possibility that α-catenin or the α-catenin binding partners vinculin, α-actinin and Formin-1 (reviewed in Kobielak and Fuchs, 2004) act as crucial signalling mediators between E-cadherin and MAL-SRF upon Ca^{2+} withdrawal.

Furthermore, p120-catenin is unlikely to mediate MAL-SRF regulation upon junction dissociation, because its membrane-proximal binding site in E-cadherin was maintained in all fusion proteins, and p120-catenin was indeed properly localised to the epithelial junctions of the stable transfectants (Fig. 5; supplementary material Fig. S3). Similarly, cortical F-actin staining was restored,

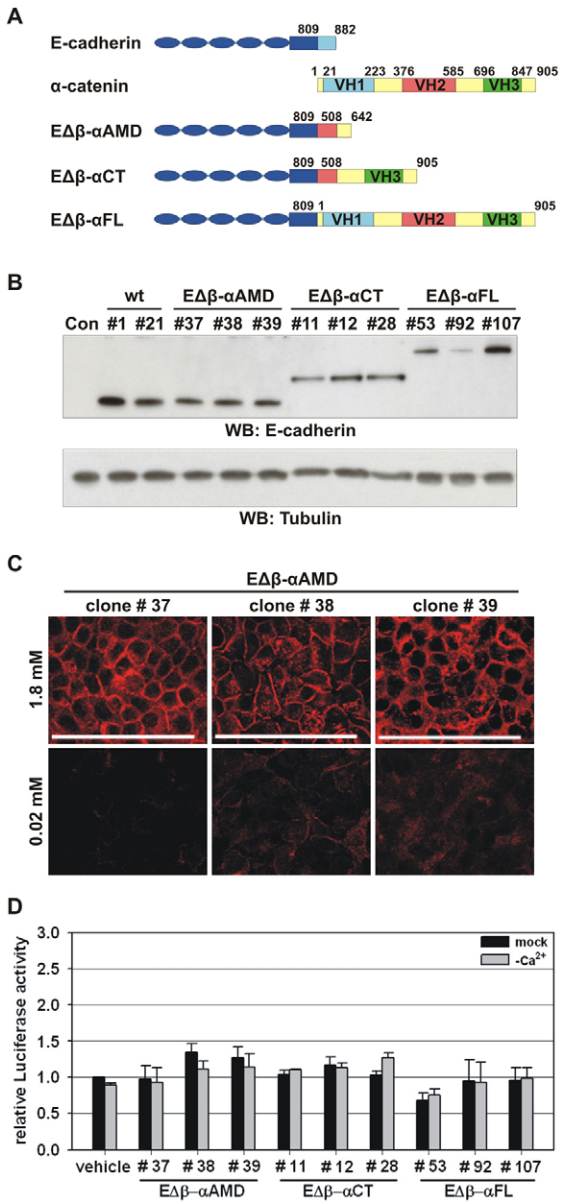

Fig. 4. E-cadherin–α-catenin fusion proteins do not activate MAL-SRF. (**A**) Scheme of human E-cadherin, α-catenin and the constructed fusion proteins. Numbers indicate amino acid residues. (**B**) Expression level of the generated fusion proteins in stably transfected clonal AGS cells as determined by western blotting. (**C**) The minimal adhesion modulation domain fused to E-cadherin (EΔβ–αAMD) localises to restored epithelial junctions. Cells were grown in a confluent monolayer for 72 hours prior to the Ca^{2+} switch for 7 hours. Cells were fixed, stained for E-cadherin (ECCD-2) and analysed by confocal microscopy. (**D**) Cell lines expressing the E-cadherin–α-catenin fusion proteins do not activate SRF upon junctional disintegration. Three independently selected clones were transfected with the MAL-SRF reporter and reseeded to form a confluent monolayer. After 72 hours the Ca^{2+} switch was performed and 7 hours later the relative luciferase induction determined. Mock, medium exchange with 1.8 mM Ca^{2+}; $-Ca^{2+}$, medium exchange with 0.02 mM Ca^{2+}. Error bars indicate s.e.m. (*n*=3). Scale bars: 50 μm.

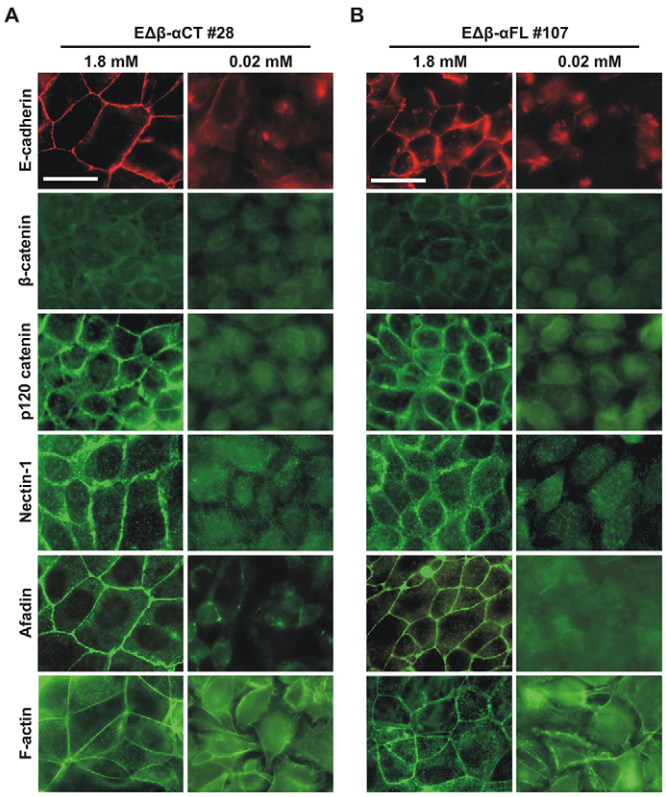

Fig. 5. E-cadherin–α-catenin fusion proteins restore epithelial junctions with nectin-afadin and p120-catenin, but not β-catenin, localisation. (**A**,**B**) The AGS clones were seeded and cultivated as described in Fig. 4D. 7 hours after Ca^{2+} switch, the cells were immunostained for E-cadherin (ECCD-2), β-catenin, p120-catenin, nectin-1, afadin and F-actin (phalloidin), and analysed by immunofluorescence microscopy. Scale bars: 25 μm.

suggesting an intact connection to the adherens junctions via the E-cadherin–α-catenin fusions. Finally, we looked for the nectin-afadin complex. Nectin-1 and afadin clearly localised to the restored cell-cell contacts, and dissociated upon Ca^{2+} switch. The lack of SRF activation, however, indicates that the nectin-afadin complex is not the AJ component that permits signalling to MAL.

By contrast, the domain necessary for β-catenin binding has been removed and substituted by the unregulatable linkage to α-catenin in the fusion constructs. β-catenin localisation was indeed strongly impaired, compared with the E-cadherin wild-type transfectants (Fig. 5; supplementary material Fig. S3). The very weak residual staining of β-catenin at the junctions might be due to its binding to the α-catenin tail of the fusions, particularly of EΔβ–αFL, which contains the VH1 domain. Together, the E-cadherin–α-catenin fusions identified the C-terminal region of E-cadherin, containing the β-catenin-binding site, as essential for MAL-SRF induction in epithelial cells.

Discussion

In previous studies we and others showed that the dissociation of epithelial cell-cell contacts facilitates transcriptional activation via Rac, G-actin, MAL and SRF (Fan et al., 2007; Busche et al., 2008). Here, we demonstrate that E-cadherin is the essential junctional component to trigger this signalling cascade. We are able to exclude tight junctions as the molecular sensor, because ZO-protein-deficient cells, which still form E-cadherin-mediated cell contacts, fully activated MAL-SRF and its endogenous targets. Vice versa, E-cadherin-deficient cells, which form basic tight

junctions, do not activate MAL-SRF. By contrast, re-expression of E-cadherin in these cells restored a functional signalling pathway and MAL-dependent transcription. Cell lines stably expressing several truncated E-cadherin–α-catenin fusion proteins, however, did not activate transcription despite restored junctional complexes, leading to the identification of the C-terminal 73 amino acids in the cytoplasmic tail of E-cadherin as being essential for signal transduction.

The results further suggest that the nectin-afadin complex, another AJ component, seems to be dispensable for MAL-SRF activation upon epithelial junction dissociation. Intracellularly, neither E-cadherin binding to p120-catenin nor its covalent linkage to full-length α-catenin is sufficient for proper SRF regulation. The latter experiment suggests that the physical integrity of epithelial cell contacts and the inducibility of MAL-SRF can be uncoupled, and point towards a crucial role of the C-terminal tail of E-cadherin during this process.

The analysis of our immunofluorescence micrographs shows that the junction formation mediated by wild-type E-cadherin correlated with a relocation of MAL into the cytoplasm. This indicates that engaged E-cadherins regulate the localisation of MAL via their β-catenin-binding site. Whether the transcriptional activity of MAL-SRF is also inhibited by engaged E-cadherins remains unclear at this stage, because the basal levels of the SRF reporter and endogenous target genes are only slightly reduced. Along this line, it was previously shown that not MAL localisation but its dissociation from the inhibitory complex with monomeric actin is the essential step for activation (Vartiainen et al., 2007). We therefore suspect that G-actin is bound to MAL even in the nuclei of AGS cells lacking wild-type E-cadherin in both basal and low Ca^{2+} conditions. Conversely, the repressive G-actin–MAL complex might dissociate upon E-cadherin disengagement, as we previously showed in MDCK cells (Busche et al., 2008).

Intriguingly, we showed that engaged E-cadherin inhibited serum responsiveness, whereas cells deficient for AJs (but not TJs) exhibit MAL-SRF activation upon serum stimulation (Fig. 3E). We thus speculate that an intracellular crosstalk between serum-dependent and E-cadherin-dependent pathways exists, and that mutual positive signals are required for proper MAL-SRF regulation in epithelial-derived cells. In line with this, cells expressing truncated or no E-cadherin failed to activate MAL-SRF signalling upon Ca^{2+} withdrawal even in the presence of serum, whereas restored MAL activation in cells re-expressing E-cadherin was not observable in serum-free conditions. Further experiments are needed to clarify this crosstalk in order to understand why epithelial cells are largely refractory to serum stimulation.

Together, our work has identified the junctional component that acts as a molecular sensor for dissociation-induced transcription by MAL and SRF. The C-terminal region of E-cadherin, containing the β-catenin-binding site, seems to be essential for MAL-SRF induction. To this end, this result allows several interpretations that are not mutually exclusive: first, β-catenin binding to E-cadherin could be directly required for MAL-SRF regulation. Second, the dynamics of the interaction with α-catenin and the cytoskeleton could be a prerequisite for the dissociation of the repressive G-actin–MAL complex. Third, an as-yet-unknown binding partner of the C-terminal E-cadherin tail could be involved in MAL-SRF activation upon epithelial disintegration. We propose that the cytoplasmic tail of E-cadherin, and its functional interactions with the Rac GTPase, transmit the signal from the dissociating cadherins to actin, MAL and SRF. How this precisely occurs remains to be investigated.

Materials and Methods

Plasmids and cells

Human wild-type E-cadherin in pEGFP-N2 was a gift from Birgit Luber (TU Munich, Germany) and human α-catenin in pOBT7 was obtained from RZPD (Berlin, Germany). The following constructs were subcloned into pcDNA3: E-cadherin wt (1-882); EΔβ–αAMD (E-cadherin 1-809, α-catenin 508-642); EΔβ–αCT (E-cadherin 1-809, α-catenin 508-905); EΔβ–αFL (E-cadherin 1-809, α-catenin 1-905). EpH4, *ZO-1*$^{-/-}$ and *ZO-1*$^{-/-}$/*ZO-2*^{kd} cells were generously provided by Sachiko Tsukita (Osaka University, Japan) and previously characterised in detail (Umeda et al., 2006). AGS cells were a gift from Thomas Meyer (MPI of Infection Biologie, Berlin, Germany). The Ca^{2+} switch was performed as described (Busche et al., 2008).

Immunofluorescence microscopy

The mouse monoclonal anti-MAL antibody (1A11), which specifically recognises MRTF-A, was raised against GST-MAL (amino acids 2-261). Immunofluorescence microscopy of MAL, F-actin and DNA was performed as described (Busche et al., 2008), with the following staining conditions: MAL 1A11, 1:5; Alexa-Fluor-546–phalloidin (Invitrogen), 1:200; DAPI (Sigma). For all other immunostainings the cells were fixed and permeabilised in methanol at –20°C for 2 minutes, and were blocked with 10% FCS, 1% gelatine, 0.05% Triton X-100 in PBS. Staining conditions were as follows: afadin (Sigma-Aldrich), 1:100; β-catenin (BD Biosciences), 1:100; wild-type E-cadherin (clone 36, BD Biosciences), 1:500; truncated E-cadherin (ECCD-2, Calbiochem), 1:100; p120-catenin (BD Biosciences), 1:100; Nectin-1 (Abcam), 1:100; ZO-1 (Zymed), 1:100. Image acquisition was described previously (Busche et al., 2008).

Transfections and luciferase reporter assay

Transfections were carried as described before (Busche et al., 2008). To generate AGS cells stably expressing the above described constructs, transfected cells were selected and expanded at 500 μg/ml G418 and further maintained without antibiotic. Before further treatment, EpH4, *ZO-1*$^{-/-}$ and *ZO-1*$^{-/-}$/*ZO-2*^{kd} cells were grown for 24 hours, AGS and AGS cells stably expressing wild-type E-cadherin were grown for 48 hours, and AGS cells stably expressing E-cadherin–α-catenin fusion constructs were grown for 72 hours. For the Ca^{2+} switch, the medium was exchanged to normal Ca^{2+} medium (1.8 mM) as control or to low Ca^{2+} medium (0.02 mM). Prior to the serum stimulus (15% FCS final concentration), the cells were starved for 24 hours in medium containing 0.2% FCS. At 7 hours after switch and/or stimulus, the cells were lysed. Luciferase reporter assays have been described (Posern et al., 2002). Protein expression was determined by western blotting using anti E-cadherin (clone 36, BD Bioscience) and anti-tubulin (Sigma-Aldrich) antibody. The Rac pull-down assay using a GST-Pak-CRIB fusion protein was performed as described previously (Busche et al., 2008).

Quantitative RT-PCR

Quantitative RT-PCR with cells was carried out as described (Busche et al., 2008), except that the cDNA quantitation was carried out on a StepOnePlus instrument (Applied Biosystems) according to the manufacturer's instructions. Additional primers were: *tjp2*: 5′-CAGAATGCGAGGATCGAAAT-3′ (forward), 5′-GTCACTGC-CGTAGCTTCCTC-3′ (reverse); *alas1*: 5′-CTGCAAAGATCTGACCCCTC-3′ (forward), 5′-CCTCATCCACGAAGGTGATT-3′ (reverse). Normalisation was to the *hprt* or *alas1* housekeeping genes for mouse or human cell lines, respectively.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/16/2803/DC1

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