RESEARCH PAPER

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Cadherin juxtamembrane region derived peptides inhibit TGFβ1 induced gene expression

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Bioactive peptides in the juxtamembrane regions of proteins are involved in many signaling events. The juxtamembrane regions of cadherins were examined for the identification of bioactive regions. Several peptides spanning the cytoplasmic juxtamembrane regions of E- and N-cadherin were synthesized and assessed for the ability to influence TGFβ responses in epithelial cells at the gene expression and protein levels. Peptides from regions closer to the membrane appeared more potent inhibitors of TGFβ signaling, blocking Smad3 phosphorylation. Thus inhibiting nuclear translocation of phosphorylated Smad complexes and subsequent transcriptional activation of TGFβ signal propagating genes. The peptides demonstrated a peptide-specific potential to inhibit other TGFβ superfamily members, such as BMP4.

Introduction

Proteins do not always interact through large ordered globular domains, but there is growing evidence of the involvement of short motifs in those interactions.[1-3] Short linear motifs are regions of typically less than ten amino acids in length, usually with less than five defined positions, which function as recognition sites for protein modification, as cleavage sites or as targeting motifs for subcellular localization.[1,4] Due to their short length they provide a small interface with their protein partners, allowing the formation of low affinity interactions with them. The formation of weak links makes them ideal for regulating interactions that need to be formed and disrupted easily.[3] Short linear motifs tend to reside inside disordered regions of proteins. The attributes of known functional motifs, such as their skewed amino acid composition, their placement in disordered regions and their tendency to evolve convergently,[5] allowed the development of bioinformatics tools that predict potentially novel active motifs.[6-12] In a previous in silico analysis we showed that disordered regions tend to reside in the juxtamembrane region of transmembrane proteins potentially accommodating linear motifs.[13] A family of transmembrane proteins, cadherins, was predicted to contain potential bioactive regions close to the membrane at their cytoplasmic side. Cadherins mediate Ca^{2+}-dependent, homophillic, cell-cell interactions, called adherens junctions.

In this study, we set off to predict bioactive peptides on the juxtamembrane regions of cadherins, and as loss of adherens junction is one of the consequences of TGFβ1 signaling, we wanted to investigate the role of these peptides on TGFβ1 signaling. Such functional peptides could give insights into potential new therapeutic approaches to targeting signaling in cancer. The TGFβ1 signaling pathway involves several protein motif-protein interactions,[14-16] commencing extracellularly with binding of growth factor to the type I and type II kinase receptors on the cell surface. The assembly of type I and type II TGFβ1 receptors into a complex requires the recognition and the phosphorylation of the GS motif in the intracellular part of the type I receptor by the type II receptor, which activates the receptor I kinase.[17] Another signaling step that requires the presence of a motif involves the phosphorylation of Smads in the C-terminal motif SSxS by the receptor complex.[18] Phosphorylated Smad2 and Smad3 form complexes with Smad4 and translocate to the nucleus where they bind many transcriptional factors modulating the expression of genes that propagate the TGFβ1 signal.[19,20]

Genes whose expression is modulated through TGFβ1 signaling include those contributing to the development of epithelial to mesenchymal transition (EMT), such as E- and N-cadherin[21] and to the generation of fibrosis, such as connective tissue growth factor (CTGF).[22] TGFβ1 also induces the expression of elements of the Notch signaling pathway, such as Jagged.[23] EMT is an evolutionary conserved process during embryogenesis that provides new embryonic tissue and specific cell lineages, like mesoderm, which later in development generate epithelial

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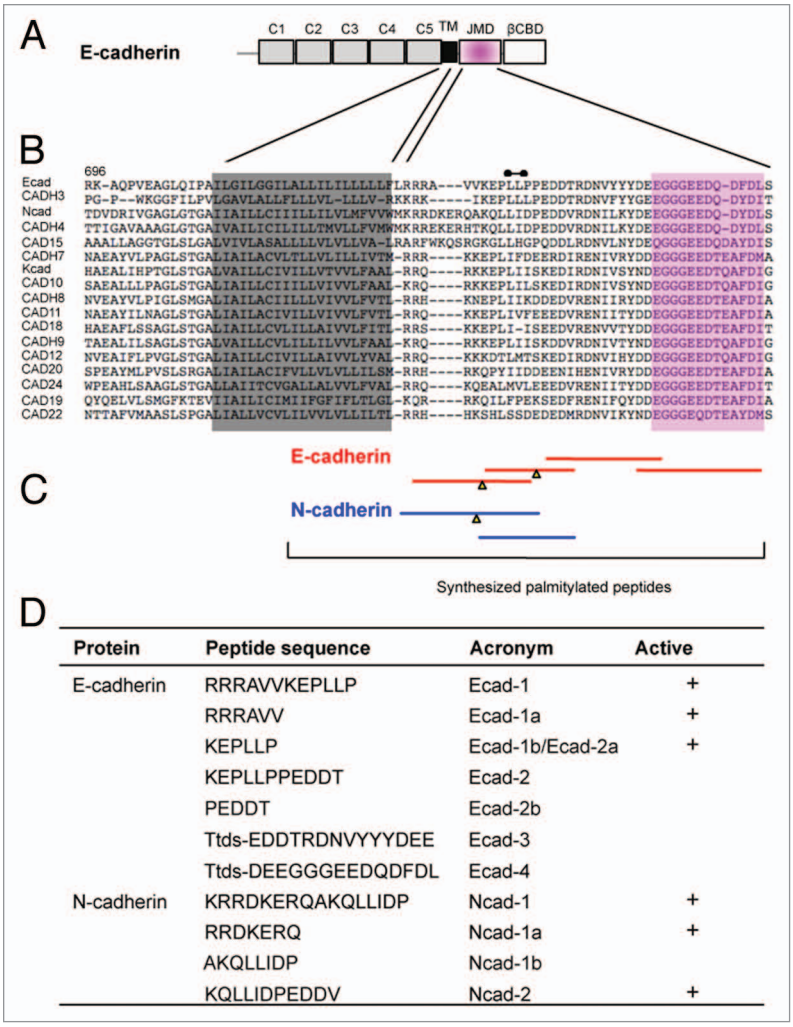

Protein	Peptide sequence	Acronym	Active
E-cadherin	RRRAVVKEPLLP	Ecad-1	+
	RRRAVV	Ecad-1a	+
	KEPLLP	Ecad-1b/Ecad-2a	+
	KEPLLPPEDDT	Ecad-2	
	PEDDT	Ecad-2b	
	Ttds-EDDTRDNVYYYDEE	Ecad-3	
	Ttds-DEEGGGEEDQDFDL	Ecad-4	
N-cadherin	KRRDKERQAKQLLIDP	Ncad-1	+
	RRDKERQ	Ncad-1a	+
	AKQLLIDP	Ncad-1b	
	KQLLIDPEDDV	Ncad-2	+

Figure 1. (A) Graphical presentation of human E-cadherin protein. C1-C5: extracellular cadherin domain, TM: transmembrane region, JMD: Juxtamembrane region, βCBD: β-catenin binding domain. (B) Multiple Sequence alignment of human Cadherin proteins. The region around the membrane is shown. The transmembrane region and the catenin p120 binding site are highlighted in gray and magenta, respectively. The dileucine (LL) endocytic motif is overlined. Peptide sequences synthesized based on E-cadherin and N-cadherin are highlighted below the multiple sequence alignment (C) and the sequences are shown on the table (D). Yellow triangles on pane C show the point where Ecad-1, Ecad-2 and Ncad-1 peptides were split to generate the shorter peptides. Peptides that inhibit TGFβ responses are labeled as active on the table (D). Ttds: 1-amino-4,7,10-trioxa-13-tridecanamine succinimic acid.

organs, such as kidney through mesenchymal to epithelial transition.[24,25] EMT activation in differentiated epithelial cells is linked with adult pathologies. There is emerging evidence of EMT's role in the generation of cancer cells with stem cell-like characteristics[26,27] and in the formation and accumulation of fibroblasts in kidney fibrosis.[22,28] TGFβ1 stimulation of CTGF expression results in the synthesis and the accumulation of extracellular matrix (ECM), leading to tissue fibrosis.[29] Notch signaling pathway is involved in the development of many tissues and organs through the regulation of cell proliferation, survival, apoptosis and differentiation.[30]

In the present study we identified peptides in the juxtamembrane region of E and N-cadherin that inhibit TGFβ1 responses at both the level of gene and protein expression. We identified shorter sub-peptides retaining the inhibitory function and mapped the active residues in those peptides.

Results

In silico identification of conserved motifs in juxtamembrane region of cadherins

The cadherin cytoplasmic juxtamembrane region influences function by supporting the accumulation of cadherins in clusters, a prerequisite for proper adherens junction formation.[31] Experimentally verified functional short linear motifs tend to reside inside locally disordered regions.[32,33] We sought to identify sites in human E- and N-cadherin protein sequences that are characterized simultaneously by high disorder and an increased relative local conservation in orthologous proteins. In general, short motifs are less conserved than structured domains. They are, however, relatively conserved compared with their adjacent residues.[34] The conservation of a few residues in an otherwise non-conserved environment may sometimes be an indication of a functional role. The Relative Local Conservation (RLC) of a disordered residue was calculated,[2] by comparing its conservation to the background distribution of adjacent disordered residues within the sequence. The in silico analysis identified groups of residues in the intracellular juxtamembrane region of E- and N-cadherin as relatively conserved and disordered (**Figs. S1 and 2**). Multiple sequence alignment of various human cadherins (**Fig. 1**) showed that cadherins are fairly conserved in their juxtamembrane region especially within the p120 catenin binding site (**Fig. 1**). The association of p120 catenin with the juxtamembrane region of the cadherin cytoplasmic tail is crucial for the surface stability of the cell-cell adhesion complexes.[35] However, there are other conserved residues closer to the membrane, whose functional roles have not been as clearly elucidated. We designed and synthesized overlapping peptides based on the E- and N-cadherin sequence (**Fig. 1**). The peptides were palmitylated at the N-terminus to facilitate tethering of the peptides to the plasma membrane.[36-39] A Ttds (1-amino-4,7,10-trioxa-13-tridecanamine succinimic acid) linker between the palmitic acid and the peptide sequence was used in Ecad-3 and Ecad-4 peptides to mimic partially the distance of the parent sequence from the plasma membrane. The peptide naming convention we adopted here was to number the peptide closest to the membrane as peptide 1 (e.g., Ecad-1), with increasing peptide numbers corresponding to further distance from the membrane. Subsequent sub-peptides of the initially investigated peptides were then labeled with the suffix a or b

in addition to the name of the larger initial peptide (**Fig. 1**). All the peptides were C-terminally amidated.

Peptides from juxtamembrane region of cadherins inhibit TGFβ1 induced gene expression in human kidney epithelial cells

We evaluated the effect of TGFβ1 on kidney cell gene expression by treating human tubular epithelial cells (HK-2) with TGFβ1 for 24 and 48 h and using various TGFβ1 doses to stimulate characteristic responses (**Figs. S3 and 4**). Induction of the TGFβ1 pathway was indicated by increased Jagged (Jagged 1), N-cadherin and CTGF expression, and decreased E-cadherin expression. Real-time TaqMan PCR was performed using probes specific for each marker. An increase in expression of all three genes, Jagged, N-cadherin and CTGF, and a decrease in E-cadherin expression were evident 24 h after TGFβ1 stimulation of the cells (**Fig. S3**). A dose of 5 ng/ml of TGFβ1 was sufficient to induce the response (**Fig. S4**). Thus, we justified using 24 h as a time point and 5 ng/ml as a dose for TGFβ1 treatment in all the experiments in the present study.

In order to investigate whether the synthesized cadherin palmitylated peptides were able to influence the TGFβ1 response, HK-2 cells were pre-incubated with the relevant peptides prior to TGFβ1 stimulation. Interestingly, cells that were pre-incubated with Ecad-1, Ncad-1 and Ncad-2 peptides suppressed the expected upregulation of both Jagged and N-cadherin upon TGFβ1 stimulation (**Fig. 2**). The expression of both markers was reduced to normal untreated levels. Ecad-3 and Ecad-4 peptides did not show any noteworthy inhibitory effect on TGFβ1 response. Palmitic acid had no effect on TGFβ1 response (**Fig. 2**), nor did palmitic acid connected to a Ttds linker (data not shown).

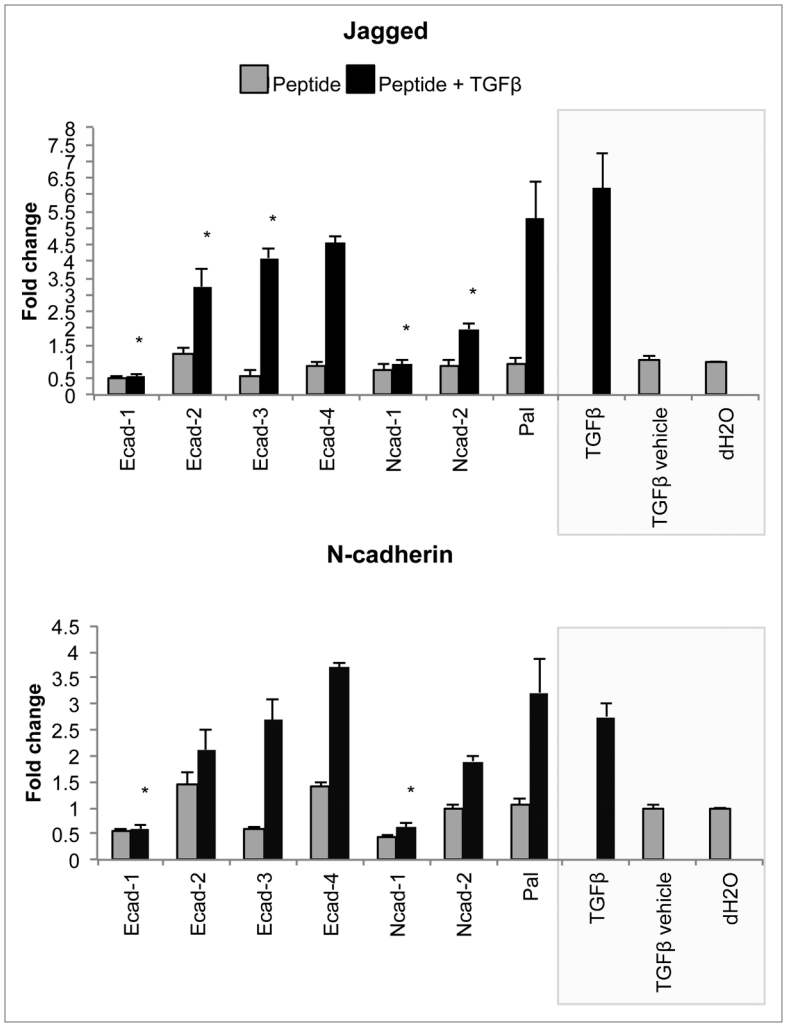

Figure 2. Effect of peptides on the expression of Jagged and N-cadherin. A dose of 50 μM of peptide was used. (Pal: Palmitic acid). *$P < 0.05$, Student's unpaired *t* test compared with TGFβ treatment alone, n = 4 per group.

When the peptides were added in the absence of TGFβ1, they failed to influence significantly Jagged and N-cadherin expression (**Fig. 2**).

Identifying bioactive regions of the cadherin peptides

As shown above, Ecad-1 peptide acts as a potent inhibitor of TGFβ1 responses and Ecad-2 peptide has a minor inhibitory effect. Both of those peptides derive from the juxtamembrane region of E-cadherin and they share an overlapping sequence (KEPLLP) (**Fig. 1**). We hypothesized that there is a primary sequence present in one or both of the peptides that drives the observed inhibitory effect, and we synthesized palmitylated and C-terminally amidated peptides for each half of each of the Ecad-1 and Ecad-2 peptides (**Fig. 1**). We examined the effect of those short peptides on Jagged and N-cadherin transcripts (**Fig. 3**). The two short peptides for Ecad-1 peptide (Ecad-1a and Ecad-1b), both effectively inhibited TGFβ1 response similarly to the full Ecad-1 peptide. Ecad-2b peptide did not show any inhibitory effect. As the Ecad-1 and Ecad-2 peptides are overlapping, Ecad-1b and Ecad-2a peptides correspond to the same sequence (KEPLLP), and they are only shown once, as Ecad-1b. The fact that both Ecad-1a and Ecad-1b peptides but not Ecad-2b peptide are capable of inhibiting the TGFβ1 response indicates that the observed effect derives from a sequence that is proximal to the membrane.

Similarly, for Ncad-1 peptide, Ncad-1a peptide that is closer to the membrane appeared to hinder TGFβ1 effect, whereas the more distant portion Ncad-1b was not effective (**Fig. 4**). Interestingly, Ecad-1b and Ncad-1b peptides derive from the same homologous region of cadherins and they both possess the dileucine endocytic motif (LL). The fact that those peptides have different effects suggests that the charge or other general properties of the flanking residues are crucial for function.

According to the dose-response experiments we conducted (**Figs. S5–8**) all peptides behave in a dose-dependent manner.

Mapping critical residues within the Ecad-1b peptide

Ecad-1b peptide is able to inhibit TGFβ1 responses and contains the dileucine endocytic motif (LL), which is involved in clathrin mediated E-cadherin internalization.[40,41] We wished to examine if the presence of the dileucine motif is responsible for

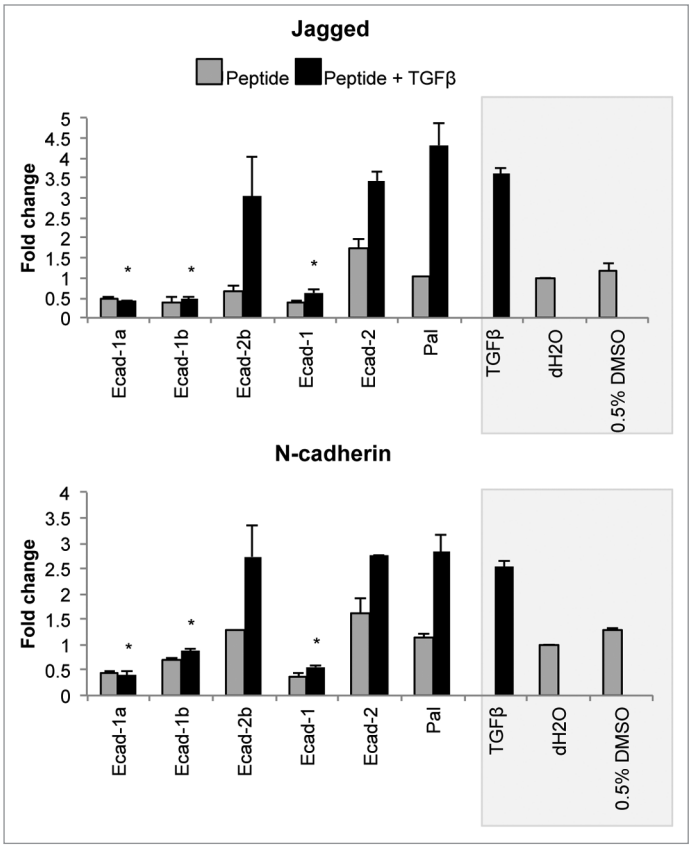

Figure 3. Effect of deletion peptides of Ecad-1 and Ecad-2 on the expression of Jagged and N-cadherin. A dose of 50 μM of peptide was used. Note that as Ecad-1 and Ecad-2 are peptides with overlapping sequence, deletion peptides Ecad-1b and Ecad-2a depict the same sequence (KEPLLP) and thus only the Ecad-1b peptide is shown on the graph. (n = 4). The Ecad-2b peptide was reconstituted in 0.5% DMSO (Dimethyl sulfoxide). (Pal: Palmitic acid). *$P < 0.05$, Student's unpaired *t* test compared with TGFβ treatment alone, n = 4 per group.

the TGFβ1 response inhibition and we synthesized palmitylated peptides with several residues substituted to alanine (A) or glutamic acid (E) (**Fig. 5**). Replacing the first leucine of the LL motif to alanine resulted in loss of inhibition. Mutation of the second leucine did not influence the peptide effect, indicating that the first leucine of the motif is important. Replacing the positive lysine (K) with neutral alanine (A) did not reduce bioactivity. The peptide partially lost activity when lysine was replaced with a negatively charged glutamic acid. These data suggest that the effect of the Ecad-1b peptide is likely to derive in part from at least one residue of the dileucine motif and that a presence of a neutral or a positive charge at the N-terminus is important.

Cadherin peptides influence primary Smad dependent TGFβ1 signaling

We further corroborated the effect of the two peptides with the strongest inhibitory effect on TGFβ1 response, Ecad-1, Ecad-2 and Ncad-1 peptides, at the protein level.

Binding of TGFβ1 ligand induces the assembly of the type I and type II serine/threonine kinase receptors to the TGFβ1 receptor complex. The type II receptor phosphorylates type I in the juxtamembrane region, which is an essential step for TGFβ1 signaling.[14] The activated receptor propagates the signal intracellularly through phosphorylation of Smad proteins. Phosphorylation of Smad3 is among the first phosphorylation steps in this signal propagation.[42] Interestingly, the expected increase of Smad3 phosphorylation after TGFβ1 stimulation was inhibited by Ecad-1 and Ncad-1 peptides and to a lesser extent by Ecad-2 peptide, whereas total Smad3 levels remained unaffected (**Fig. 6**). These data suggest that the peptides may act at the very early stages of the pathway, likely proximal to the TGFβ receptor.

Figures 6 and **S9** also shows that several markers of TGFβ1 responses were upregulated upon TGFβ1 stimulation. Smad3 levels were used as an internal loading control. This TGFβ1 induced protein overexpression was abolished after pre-incubation of the cells with the cadherin peptides. Ecad-1 and Ncad-1 were the most potent inhibitors, successfully suppressing Jagged, N-cadherin and fibronectin upregulation (**Figs. 6** and **S9**). Ecad-2 peptide inhibitory effect was more prominent in fibronectin expression, having no significant effect on Jagged and N-cadherin expression. These results suggest that the cadherin peptides inhibit the expression of mesenchymal markers, keeping the cells in the epithelial state even after TGFβ1 stimulation.

TGFβ1 is part of a superfamily of transforming growth factors that include activins and bone morphogenetic proteins (BMPs). We investigated the effect of the peptides in BMP4 pathway using western blot analysis for the phosphorylation levels of Smad-1/5/8 (**Fig. S9**). We observed that Ecad-1 peptide inhibited BMP4 induced phosphorylation of Smad-1/5/8, similarly to Noggin, a BMP4 inhibitor. Noggin is a small glycoprotein (32 kDa), which binds to BMP4 and antagonizes BMP signaling by blocking BMP4 receptor interaction.[43] Ncad-1 peptide showed less potency to inhibit BMP4 responses compared with its effect on TGFβ1. Therefore, we suggest that the peptides probably affect signaling by all TGFβ superfamily receptors but with an intensity that is peptide dependent or specific.

Discussion

In the present study we tried to identify functional motifs in the cytoplasmic regions of E-cadherin and N-cadherin. Cadherins are Ca^{2+}-dependent molecules present on the cell surface that are involved in adherens junctions and intercellular recognition. The extracellular segments of E-cadherins are involved in intercellular homophilic binding with E-cadherins present on neighboring cells, whereas the intracellular parts interact with catenins, such as p120 catenin, β-catenin and α-catenin. The latter interactions connect the intracellular segments of E-cadherins with actin, thus controlling cytoskeletal changes. The interactions that involve cadherin-catenin complexes constitute the adhesion dependent functions of cadherins. Other roles of cadherins do not require adhesion and they form the adhesion independent functions (reviewed in ref. 44). Cadherins may interact with

growth factor receptors, in a ligand-independent manner, regardless of the formation of adherens junction.[45]

We provided data that our peptides inhibited TGFβ1 responses at the gene expression and at the protein level. Of the tested peptides those derived from sequences most proximal to the membrane were more potent inhibitors of the TGFβ1 responses. Ecad-1 and Ncad-1 peptides inhibited Smad3 phosphorylation, as well as other TGFβ1 responsive proteins, suggesting that the peptides inhibit the TGFβ1 signaling at the initial steps of the pathway. As expected, Jagged1 levels were also downregulated. Jagged1 increase upon TGFβ1 treatment is dependent on Smad3 phosphorylation.[23] The transcription of Jagged1 requires crosstalk between TGFβ1 and Wnt/Notch signaling.[23] Activation of Notch signaling has been shown to regulate EMT genes in mammary and kidney epithelial cells,[46] propagating in this way the TGFβ1 response. Thus, the peptide induced decrease in Jagged1 expression could potentially influence the Notch pathway indirectly. Prevention of Smad3 phosphorylation leading to inhibition of TGFβ1 responses has been reported after direct interaction of Akt with Smad3.[47,48]

However, we were not able to identify if this inhibition was due to a direct interaction of the peptides with Smad3, or more likely as a result of indirect interaction through other proteins. It is well established that inhibition of TGFβ1 signaling is associated with a sustained epithelial phenotype, highlighted by the maintenance of a stable adherens junction mediated by E-cadherin.[21,49] Therefore, we discuss below possible ways that the peptides could assist the cells to retain their epithelial phenotype.

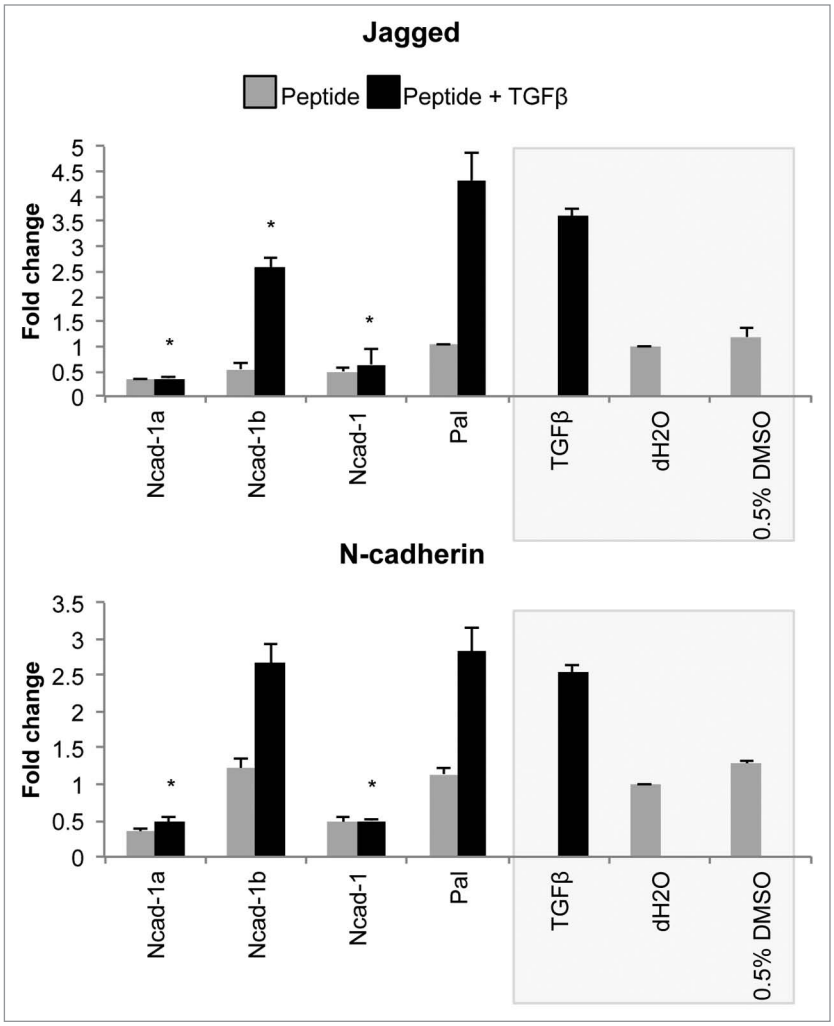

Figure 4. Effect of deletion peptides of Ncad-1 on the expression of Jagged and N-cadherin in the presence and in the absence of TGFβ. A dose of 50 μM of peptide was used. (n = 4). The Ncad-1b peptide was reconstituted in 0.5% DMSO. (Pal: Palmitic acid). *$P < 0.05$, Student's unpaired *t* test compared with TGFβ treatment alone, n = 4 per group.

The bioactive juxtamembrane cadherin-derived peptides may mimic the parent protein that they are derived from. The juxtamembrane region of E-cadherin has been previously shown to be crucial for the accumulation of E-cadherins in clusters and for the formation of tight adherens junctions, involving binding of p120-catenin and β-catenin to the cadherin cytoplasmic tail.[35,50] Our membrane tethered peptides (via N-terminus palmitic acid) do not span the known binding region of either the p120-catenin or the β-catenin, but they derive from a region closer to the membrane that forms a dynamic interaction with p120-catenin.[51] If this short region can independently interact with p120-catenin, it could provide a reason for the p120-catenin to remain in the membrane proximal environment even after a TGFβ1 induced E-cadherin depletion. Non-cadherin-associated-p120 catenin has been shown to exhibit GTPase effects, inhibiting RhoA and activating Rac1,[52-54] and thus facilitates increased cytoskeletal changes and cell motility, usually seen upon TGFβ treatment. Therefore, it would be interesting to examine the possibility that the presence of those peptides reduce the free p120 catenin population in the cell, thus causing reduced TGFβ responses.

Of the shorter active peptides, the Ecad-1b peptide (KEPLLP) appeared potent to inhibit TGFβ1 responses even in lower doses. The Ecad-1b peptide contains the dileucine (LL) motif, which plays a role in the intracellular accumulation of E-cadherin and its targeting to lysosomes for degradation.[40] The dileucine motif is recognized by the AP2 clathrin adaptor complex and targets the protein that carries the motif to clathrin-coated vesicles.[55] E-cadherin constructs that had the dileucine motif substituted to Alanine escaped internalization and remained on the cell surface.[40] The fact that a dileucine containing peptide, like the Ecad-1b, is able to inhibit TGFβ1 responses could potentially be attributed to action of the peptide keeping the endocytic machinery of the cell occupied, allowing the E-cadherin to escape internalization. When we replaced the Leucine to Alanine the peptide showed reduced activity, whereas substitution of the second Leucine to Alanine did not affect the peptide function.

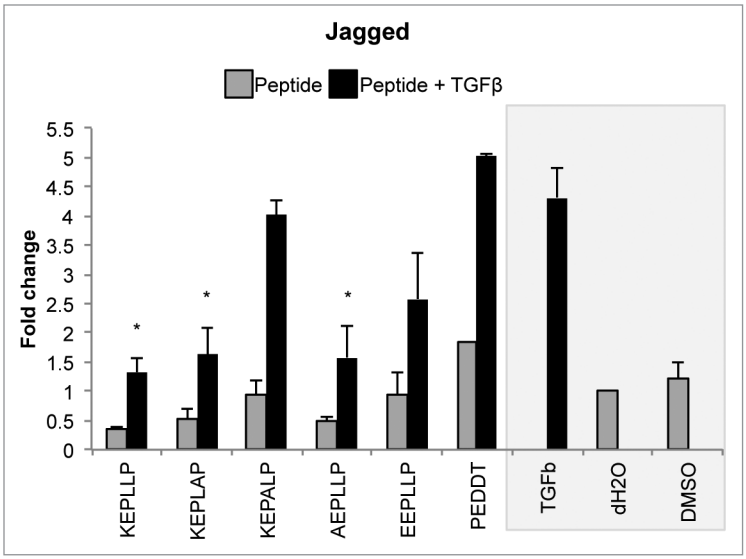

Figure 5. Effect of Ecad-1b (KEPLLP) peptide and its control peptides on the expression of Jagged. A 12.5 μM dose of peptide was used. Replacing the second leucine of the endocytic dileucin motif does not affect the function of the peptide, whereas an alanine substitution of the first leucine hinders the ability of the peptide to inhibit TGFβ. (Pal: Palmitic acid). *$P < 0.05$, Student's unpaired *t* test compared with TGFβ treatment alone, n = 4 per group.

Since functionality does not require conservation of both of these hydrophobic amino acids typically required for AP2 binding,[55] we suspect that the mechanism of action is therefore more likely to rely on another mechanism.

Methods

Bioinformatics analysis

Protein sequences of human E- and N-cadherin were analyzed using the Gopher algorithm (http://bioware.ucd.ie) for the identification of orthologous proteins in metazoan proteins contained in the EnsEMBL sequence database of sequenced genomes. Gopher used Muscle[56] to generate multiple sequence alignment of each human protein with each orthologs. These alignments were then used for the calculation of Relative Local Conservation (RLC) score.[2] The software used IUPred[57] to estimate the disorder score for each residue in the human proteins. For the identification of conserved residues among cadherins a multiple protein sequence alignment was generated with Muscle.

Cell culture

Human kidney epithelial cells were cultured at 37 °C in a humidified atmosphere of 95% air/5% CO2. Culture

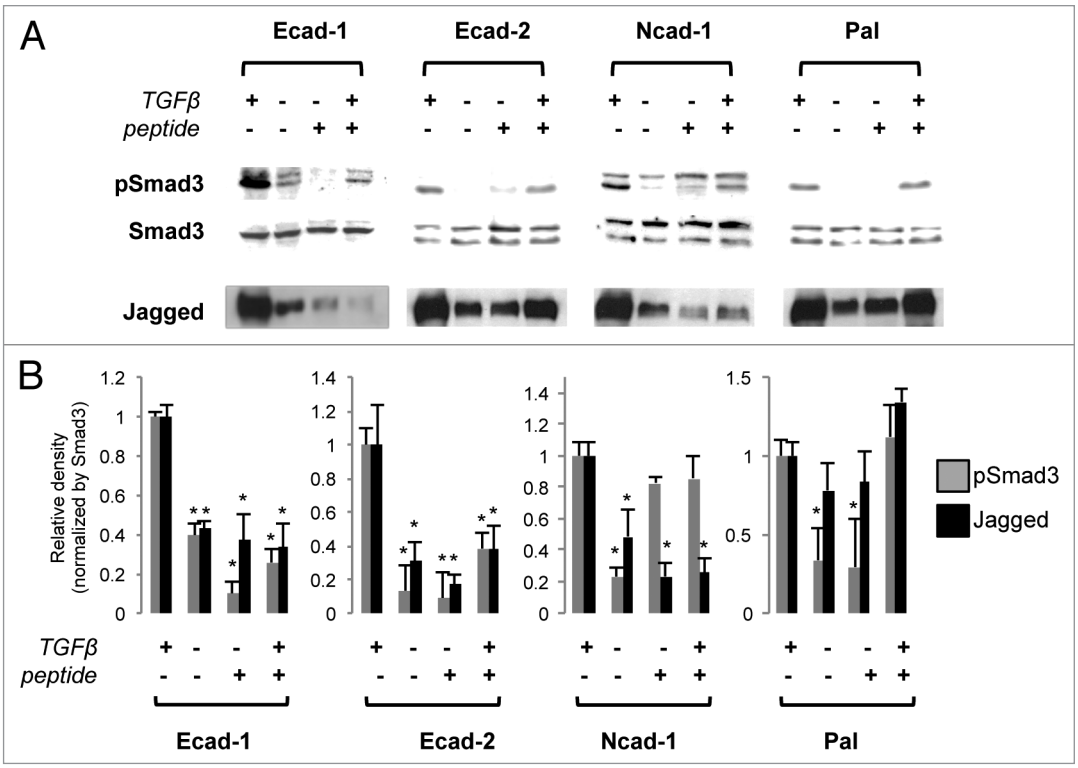

Figure 6. (A)Effect of peptides on the protein levels of pSmad3 and Jagged. HK-2 cells were treated with 50 μM of the indicated peptides for 24 h before cells were lysed and protein extract was collected. (B) Quantification of protein bands using Smad3 as loading control. (Pal: Palmitic acid). *$P < 0.05$, Student's unpaired *t* test compared with TGFβ treatment alone, n = 3 per group.

medium contained Dulbecco's modified Eagle's medium (DMEM-F12, Sigma) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml EGF, 36 ng/ml hydrocortisone, 4 pg/ml triidothyronine and 5 μg/ml insulin-transferin-selenium solution (ITS, Sigma).

For TGFβ1 treatments, HK-2 cells were cultured in 12-well plates and treated with 5 ng/ml recombinant human TGFβ1 (R&D Systems) or vehicle for 24 h. Palmitylated peptides were added 45 min prior to TGFβ1 addition at either 50 μM, 25 μM, 12.5 μM or 1 μM.

RNA extraction and Real-time PCR of HK-2 cells

RNA was extracted using the RNeasy mini kit (Qiagen) following the manufacturer's protocol. The concentration of RNA was estimated on a NanoDrop® ND-1000 spectrophotometer and cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen). For real-time TaqMan PCR analysis specific probes for Jagged (Hs00164982_ml), N-cadherin (Hs00169953_ml), E-cadherin (Hs00170423) and CTGF (Hs00170014_ml) were used. TaqMan Universal PCR Master Mix (Applied Biosystems) was used for the reactions and samples were analyzed on an ABI Prism 7700 sequence detection system at defaults thermal cycling conditions: 2 min at 50 °C, 10 min at 95 °C and then 40 cycles of 15 s at 95 °C for denaturation and 1 min at 60 °C for annealing and extension. Results were analyzed using the ΔΔCt method and normalized to 18S rRNA levels.

Protein extraction and western blotting

For protein analysis, HK-2 cells were cultured in 10 cm petri dishes and they were harvested in RIPA buffer containing 50 mM TRIS-HCl pH 7.4, 1% (v/v) Nonidet P-40, 150 mM NaCl, 1 mM Na_2VO_4, 1mM NaF, 1mM PMSF, and 1/100 dilution of protease inhibitor cocktail (Sigma). Samples were incubated on ice for 40 min, agitating regularly. Samples were then centrifuged at 14,000 rpm at 4 °C for 12 min to remove cell debris. The determination of protein concentration was performed using the method of Bradford[58] with the protein assay concentrate (BioRad). The absorbance of the suspension at 595 nm was read in a Beckman UV/VIS spectrophotometer (DU530). Proteins were resolved using 10% SDS-PAGE for 1h at 20 mA constant current and then transferred to a polyvinylidene diflouride membrane (PVDF, Whatman) at 110 V for 60 min. Membranes were blocked with 5% (w/v) milk in TBS-T [20mM Tris-base, 137mM NaCl, 0.1% (v/v) Tween-20] for 60 min at room temperature. Membranes were incubated with primary antibodies for Jagged1, N-cadherin, fibronectin, phospho-Smad3, Smad3, phospho-Smad1/5/8 and GAPDH diluted in 5% milk in TBS-T overnight at 4 °C. The following day primary antibodies were removed by washing the membranes in TBS-T [3 × 15 min washes at room temperature (RT)] and they were then incubated with HRP-coupled anti-rabbit or anti-mouse secondary antibodies at 1:2000 dilution in 5% milk for 1 h at RT. Secondary antibodies were removed by washing with TBS-T (3 × 15 min washes at RT) and membranes were developed by incubation with enhanced chemiluminescence ECL solution (Supersignal West Dura) and exposed to X-ray film to reveal the reactive bands. Membranes were stripped by incubation with Restore PLUS Western Blot Stripping buffer (Thermo Scientific) for 15 min at RT. The stripping buffer was removed by washing with TBS-T and membranes were then re-blocked with 5% milk and re-probed with appropriate primary antibodies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Material

Supplemental materials may be found here:
http://www.landesbioscience.com/journals/BioArchitecture/article/32143/

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