Bves modulates epithelial integrity through an interaction at the tight junction

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Summary

We first identified Bves (blood vessel/epicardial substance) as a transmembrane protein that localized to the lateral compartment of the epithelial epicardium. Bves traffics to sites of cell-cell contact in cultured epicardial cells and promotes adhesion following transfection into non-adherent fibroblastic L-cells, reminiscent of a cell adhesion molecule. Currently, no function for Bves in relation to epithelial cell adhesion has been identified. We hypothesize that Bves plays a role at cell junctions to establish and/or modulate cell adhesion or cell-cell interactions in epithelial cell types. In this study, we demonstrate that Bves regulates epithelial integrity and that this function may be associated with a role at the tight junction (TJ). We report that Bves localizes with ZO-1 and occludin, markers of the TJ, in polarized epithelial cell lines and in vivo. We find that the behavior of Bves following low Ca²⁺ challenge or TPA treatment mimics that observed for ZO-1 and is distinct from adherens junction proteins such as E-cadherin. Furthermore, GST pull-down experiments show an interaction between ZO-1 and the intracellular C-terminal tail of Bves. Finally, we demonstrate that Bves modulates tight junction integrity, as indicated by the loss of transepithelial resistance and junction protein localization at the membrane following Bves knock-down in cultured cells. This study is the first to identify a function for Bves in epithelia and supports the hypothesis that Bves contributes to establishment and/or maintenance of epithelial cell integrity.

Introduction

Epithelia are tightly bound sheets of cells supported by a basal lamina that cover or line all of the body surfaces, cavities and tubes. Epithelial cells are maintained as structured and uniform sheets by cell junctions, which are multi-protein complexes that lie at cell-cell boundaries and regulate the association of neighboring cells. Cell-cell adhesion, generated by these junctions, provides the structural integrity that is critical for proper development of the embryo and for maintenance of tissues in the adult (Gumbiner, 1996). The importance of epithelial integrity and regulation of cell adhesion is underscored by the severity of anomalies, such as polycystic kidney disease, skin blistering diseases, and many types of cancer that arise from an alteration in molecules that maintain a cohesive epithelial monolayer (Amagai et al., 1991; Charron et al., 2000; Guilford et al., 1998; Stanley, 1995; Wilson, 1997).

One principle feature of epithelia is the polarization of their cells, which is mediated by junctions present along the lateral surface, including tight junctions (TJ), adherens junctions (AJ) and desmosomes (Garrod et al., 1996; Gonzalez-Mariscal et al., 2003; Nagafuchi, 2001). The two key functions of the TJ are the establishment of apical-basal polarity, to retain complexes and/or receptors within the proper membrane domain, and the creation of a water-tight seal, to prevent the undesired passage of ions or fluid through the cell layer (Balda and Matter, 2000). Primary components of TJs are transmembrane elements such as occludin (Furuse et al., 1993) and the claudins (Furuse et al., 1998) and peripheral membrane proteins ZO-1 (Stevenson et al., 1986), ZO-2 (Gumbiner et al., 1991) and ZO-3 (Haskins et al., 1998). The AJ, found immediately below the TJ, provides the core adhesive interaction between neighbors by interconnecting the actin network (Nagafuchi, 2001). E-cadherin, a transmembrane component of the AJ, maintains cell-cell adhesion through Ca²⁺-dependent homophilic binding (Adams et al., 1998a). Desmosomes provide additional mechanical strength to the cell structure by serving as anchoring sites for the intermediate filament network spanning the epithelial sheet (Gettsios et al., 2004). These three junctional networks regulate the strength of adhesion between cells, thus permitting epithelia to modulate their integrity to meet the functional requirements of the tissue or organ (Garrod et al., 1996; Provost and Rimm, 1999; Runswick et al., 2001; Schneeberger and Lynch, 2004). For example, during embryonic development, the epithelial germ layers undergo dynamic morphogenetic movements in order to complete the processes of gastrulation and neurulation (Gerhart and Keller, 1986; Marsden and DeSimone, 2003; Wallingford et al., 2002). During these processes, the adhesive nature and integrity of the epithelia are regulated such that proper tissue rearrangements occur. Understanding the
contributory role of each junctional complex in the regulation of tissue integrity is crucial to unraveling the intricacies of epithelial cell-cell interactions.

The function of each junctional complex is generally understood and numerous components have been identified (Balda and Matter, 2000; Garrod et al., 1996; Gonzalez-Mariscal et al., 2003; Gumbiner, 1996). However, additional roles for and interactions between junctional proteins are continuously revealed. Thus, the discovery of novel regulators and components of cell junctions is essential for gaining insight into the mechanism by which junctions are established during formation and establishment of epithelial integrity. In the following study, we provide evidence that Bves [also called Pop1 (Andree et al., 2000)] plays a pivotal role in the regulation of epithelial integrity and the maintenance of adhesive cell junctions. Originally identified as a heart-enriched gene product, Bves (blood vessel/epicardial substance) shows no significant homology to any known gene product but is highly conserved across species in which it has been identified (Andree et al., 2000; DiAngelo et al., 2001; Knight, 2003; Osler and Bader, 2004; Reese et al., 1999; Ripley et al., 2004; Wada et al., 2001). We have previously demonstrated that Bves is an ~48 kDa three-pass transmembrane protein that localizes to the lateral membrane of the epicardium and, when transfected, confers adhesion to non-adherent mouse fibroblast cells (Wada et al., 2001). However, the current literature on Bves and related proteins of the Popeye gene family fails to demonstrate any function for Bves in epithelia. Therefore, given the localization and adhesive nature of Bves, we hypothesize that Bves modulates epithelial integrity by its direct or indirect influence on cell cohesion and/or cell junctions. Thus, a critical step toward a comprehensive understanding of the essential nature of Bves is the demonstration of its function in the maintenance of epithelial integrity.

In the current investigation, we demonstrate that Bves localizes closely with TJ components such as ZO-1 and occludin in mature epithelia. Additionally, we determine the timeframe of junction formation/maturation during which Bves is trafficked to the membrane. Furthermore, based on striking similarities to ZO-1 following physiological challenges to cell adhesion, we predict that Bves function is coupled to its localization of the TJ. GST pull-down experiments demonstrate an interaction with ZO-1, further supporting a function at the TJ. Finally, using an antisense morpholino oligonucleotide (MO) knockdown/rescue approach, we reveal localization of the TJ. GST pull-down experiments using an antisense morpholino oligonucleotide (MO) knockdown/rescue approach, we reveal localization of the TJ. GST pull-down experiments

**Materials and Methods**

**Cells and antibodies**

Epicardial mesothelial cells (EMCs) were obtained from H. Eid and have been described previously (Eid et al., 1994; Wada et al., 2003). Madin-Darby canine kidney (MDCK) epithelial cells (from the American Type Culture Collection; ATCC), Caco-2 colon epithelial cells (a gift from R. Coffey, Vanderbilt University, Nashville, TN, USA) and 4T-1 mammary cells (a gift from L. Matrisian, Vanderbilt University) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Biowhittaker, Walkersville, MD) supplemented with 10% FBS and penicillin/streptomycin cocktail. The SV40-transformed human corneal epithelial (HCE) cells, kindly provided by K. Araki-Sasaki (Araki-Sasaki et al., 1995) were grown in serum-free keratinocyte growth medium (Invitrogen-Gibco). Standard protocols were used for cell culture and tissue sections (Osler and Bader, 2004; Wada et al., 2001). The rabbit anti-Bves antiserum (B846 at 1:200) used in this study has been described previously (Wada et al., 2001) and detects an intracellular C-terminal epitope of Bves in all species tested. Antibodies were used according to published methods and manufacturer’s recommendations and were purchased as follows: E-cadherin monoclonal (Transduction Labs), ZO-1 and occludin monoclonal antibodies (Zymed), desmosome monoclonal (Sigma), Alexa Fluor 488- and Alexa Fluor 568-conjugated secondary antibodies, phallolidin and DAPI (Molecular Probes). Antibody concentrations used for western blot analysis are as follows: B846 at 1:200, monoclonal β-actin at 1:5000 (Sigma), ZO-1 at 1:1000 (Zymed), and occludin at 1:500 (Zymed), HRP-conjugated secondary antibodies were used at 1:10,000 (Pierce).

**Immunofluorescence and electron microscopy**

Confocal image capture using a Zeiss LM-410 or LM-510 microscope was performed in part through the use of the VUMC Cell Imaging Shared Resource, and processed using MetaMorph 6.1 software (Universal Imaging Corp.). To generate samples for electron microscopy, an adult mouse was starved overnight, the small intestine was dissected, washed with PBS and perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 30 minutes at room temperature (RT). Following fixation, the intestine was dehydrated through a graded series of alcohols and embedded in Lowicryl resin. Nickel grids with thin sections were blocked in 1% bovine serum albumin (BSA) in PBS, incubated with B846 antiserum at 1:100 at 4°C overnight, and bathed with anti-rabbit 5 nm immunogold-conjugated secondary antibody (Sigma) for 1 hour at RT. Grids were fixed with 2.5% glutaraldehyde for 15 minutes and counterstained with 2% uranyl acetate for 5 minutes. PBS washes were performed between each step. Quantification of colloidal gold bead binding was performed on sections perpendicular to the cell surface. The distance of each bead from the cell surface was determined and grouped in increments of 100 nm. Controls using no primary antibody were performed and no bead labeling was detected. Experiments were performed in part through the use of the VUMC Research EM Resource.

**Glutathione bead preparation**

GST fusion proteins were generated by PCR from the C-terminal tail of Bves (aa 115-347) and the N-terminal tail (aa 1-36) by A. Wada and cloned into the pGEX bacterial expression vector. GST-N terminal Bves, a 34 kDa protein, consists of the GST tag 5’ of the extracellular N-terminal region of Bves. GST-C terminal Bves, which migrates at 66 kDa, contains the GST tag followed by the intracellular C-terminal tail. Constructs were transformed into BL21 E. coli bacterial strain and protein was induced with isopropyl-β-D-thiogalactopyranoside (IPTG), using standard methods (Amersham). Bacterial lysate was stored at −80°C until use. Preparation of GST beads for pull-down was performed as follows. A 50% slurry of glutathione-Sepharose 4B was prepared from a commercially available 75% slurry (Amersham). An aliquot of 1 ml of bacterial lysate expressing the GST fusion proteins was cleared by centrifugation (14,000 g) prior to the addition of 40 µl of 50% slurry. Cleared lysate was incubated with beads for at least 2 hours or overnight, beads were washed three times with 100 µl of PBS, and resuspended in 100 µl of PBS. Samples from all three
fractions were subjected to PAGE and colloidal blue staining and the amount of GST-bound protein used for pulldowns was equilibrated.

**GST pulldown**

MDCK cell lysate used for GST pulldown experiments were performed using the methods of Fanning et al. (Fanning et al., 1998). Briefly, cells were grown on 60 mm plates to confluence, placed on ice, and washed twice with PBS. Protein was extracted with 1 ml of extraction buffer (20 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.05% SDS, 1 mg/ml BSA, 1 mM DTT) and 100 μl of protease inhibitor (Sigma, P8340). Cells were incubated on ice for 30 minutes, scraped off the plate and centrifuged for 30 minutes at 18,000 g at 4°C. Cell lysate was removed from the pellet and retained. Lysate was preclarified by incubation with 20 μl bed volume of beads for 2 hours at 4°C, after which beads were spun down and lysate was removed. Glutathione beads bound with GST constructs were added to the lysate and incubated overnight at 4°C. Beads were washed 5 times with 100 μl PBS and bound protein was eluted with 20 μl of 1× SDS sample buffer, boiled for 3 minutes, and loaded onto an 8–10% polyacrylamide gel. Western blotting was performed using standard methods and the antibody concentrations used were as listed above.

**Immunoblotting of HCE cells**

Western blotting was performed as previously reported (Knight, 2003). Briefly, cells were harvested by trypsin treatment and resuspended in 1 ml TBS with protein inhibitor (0.5%; Roche Diagnostics, Cat. no. 1836170). Cells were disrupted by sonication which was followed by centrifugation to collect the pellet. The supernatant was collected and 20 μg total protein was separated by polyacrylamide gel electrophoresis. The samples were transferred to a polyvinylidene fluoride membrane (Immobilon-P membrane; Millipore). The membranes were probed with antibodies against Bves, β-actin and ZO-1, followed by appropriate species HRP-conjugated secondary antibodies (Pierce, Rockford, IL, USA).

**Cell culture treatments**

Low Ca²⁺ culture conditions were achieved by growing EMCs in MEM (Sigma) with 8% serum (Atlanta Biosciences) and penicillin-streptomycin cocktail. A small amount of calcium was added back to serum-free DMEM with normal Ca²⁺ levels. 5 mM EGTA for 2 hours. Control wells were switched to serum-free DMEM with normal Ca²⁺ levels. The following day, cells were incubated in serum-free DMEM chamber slides (Lab-Tek) in complete DMEM. The EMC line can support the development of a primordial monolayer even under persistent conditions of low Ca²⁺. Cells continue to grow, although more slowly, when maintained for several weeks in low Ca²⁺ medium. Experiments were performed following passages three or four in low Ca²⁺ medium. For 12-O-tetradecanoylphorbol 13-acetate (TPA) treatment of cells, MDCK or EMC cells were plated at high density (5×10⁵ cells/well) on 4-well chamber slides (Lab-Tek) in complete DMEM. The following day, cells were incubated in serum-free DMEM for 1 hour. Cells were switched to serum-free DMEM with 5 mM EGTA for 2 hours. Control wells were switched back to serum-free DMEM with normal Ca²⁺ levels. Experimental wells were treated with 100 nM TPA in DMEM for 1 hour. Cells were fixed and processed by standard methods (as referenced above). The anti-Bves morpholino oligonucleotide (MO) used in this study has been described previously (Ripley et al., 2004). HCE cells were treated with nonspecific control or anti-human Bves MOs at 3 and 5 days after cells reached full confluence, at which time TER measurements were recorded.

**Measure of transepithelial resistance**

HCE lines were seeded onto clear polycarbonate (0.4 μm) membrane cell culture inserts (Falcon, no. 35-3090) at a density of 10⁴ cells/cm². The transepithelial resistance (TER) was measured at 14 days using an epithelial voltmeter (EVOX-5A; World Precision Instruments, Sarasota, FL, USA). After the TER was obtained, polycarbonate membranes were cut away from the plastic insert and immunofluorescence staining was performed.

**Generation of chicken Bves HCE cells for rescue experiments**

In order to assign specific phenotypes to the MO knockdown of Bves, a rescue strategy was applied. A 'rescue' plasmid was generated as follows: chick Bves cDNA (Reese et al., 1999), which encodes the full length Bves protein (358 amino acids) and does not contain the MO target sequence, was cloned in frame into a neomycin-resistant expression plasmid with CMV promoter and FLAG epitope. HCE cells were transfected using Lipofectamine 2000 (Invitrogen) and selected in 20 μg/ml G418 antibiotic (Sigma). Five clones were selected based on FLAG labeling at the cell surface. The HCE cell line reported here, which stably expresses the MO-resistant chicken Bves rescue construct, is referred to as HCE-R. Two-tailed Student’s t-test statistical analyses were performed to determine statistical significance (Microsoft Excel).

**Results**

**Bves membrane localization pattern is conserved in various epithelial cells**

To determine whether Bves is a conserved component of epithelia, we analyzed its expression in several epithelial cell lines of varying origin, including several cancer lines. We detected Bves in a variety of epithelial cell types and observed a pattern that paralleled that observed in the EMCs, a rat squamous epicardially derived line (Fig. 1A). Labeling of Bves at the cell membrane was observed in all epithelial lines.

**Fig. 1. Bves expression in various epithelial cell lines. The B846 polyclonal anti-Bves antiserum labels Bves around the cell periphery of confluent monolayers of different epithelial cell lines. (A) Epicardial mesothelial cells (EMCs). (B) MDCK cells. (C) Caco-2 cells. (D) Mouse 4T-1 mammary cells. (E) HCE cells. (F) CHO cells. Arrows denote membrane labeling. Intracellular labeling of Bves in the Golgi occurs in several cell lines. Scale bar: 50 μm.**
examined (Fig. 1, arrows) including MDCK, a simple cuboidal kidney epithelium (Fig. 1B), columnar Caco-2 human colon adenocarcinoma (Fig. 1C), cuboidal 4T-1 mouse mammary tumor cells (Fig. 1D) and HCE, a stratified squamous cell line (Fig. 1E). Expression was not restricted exclusively to heart tissue or a specific type of epithelia, unlike, for example, tight junction protein JEAP, which is only endogenously expressed in various exocrine cells (Nishimura et al., 2002). While Bves appears to be weakly expressed at cell-cell contacts in HEK293 cells and NRK cells, it is not expressed in all fibroblast cell lines, for example, CHO cells (Fig. 1F). These data demonstrate that Bves is a conserved component of divergent epithelial cell types.

Bves co-localizes with tight junction markers in epithelial monolayers

We have defined Bves as an integral membrane protein distributed at cell borders (Fig. 1) that is a potential regulator of cell adhesion (Wada et al., 2001). However, it is not known whether Bves localizes to a particular junction complex within the lateral compartment. In order to assign Bves to a specific domain at the cell membrane, the subcellular distribution of Bves was compared to that of defined components of the TJ, AJ and desmosome. For this confocal microscopic investigation, we used the MDCK cell line, since it has been extensively studied and because a wide range of marker antibodies is available for analysis. Findings revealed that Bves is highly colocalized with the TJ proteins occludin and ZO-1 in an apical-lateral position within the z axis (Fig. 2A,B, arrows). Bves did not exhibit marked overlap with AJ-associated proteins E-cadherin (Fig. 2C, arrows) and β-catenin (Fig. 2D, arrows), or with desmosomal proteins (Fig. 2E, arrows). A similar pattern of colocalization between Bves and TJ proteins was observed in other epithelial lines including squamous HCE cells (Fig. 7A) and EMCs, as well as Caco-2 and HCA-7 cells, which both form a columnar epithelium (data not shown).

To extend these findings, we next determined the distribution of Bves in vivo at the cellular and ultrastructural levels using the murine intestinal absorptive epithelium as a model. This highly polarized columnar cell layer is ideal for identifying potential epithelial junction proteins because the TJ and AJ are well separated (Itoh et al., 1993). Cross sections through intestinal villi revealed a precise overlap of Bves, occludin and ZO-1 that was not mirrored by the differing distribution patterns of Bves and E-cadherin (Fig. 3A). Ultrastructural analysis using immunogold labeling of Bves on the gastric epithelium corroborated this finding (Fig. 3B). TJ strands are primarily located in the region from the base of the microvilli to 300 nm below the apical surface (Bloom and Fawcett, 1975). In images of perpendicular sections, gold beads...
were clustered in the apical TJ region (Fig. 3Ba-c). An en face view of TJ strands also demonstrates the concentration of gold bead labeling at or very near the TJ domain (Fig. 3Bd). Graphical representation of colloidal gold distribution demonstrated that the majority of beads (64%) were located within the TJ domain (Fig. 3C, red line). However, Bves is not entirely restricted to the TJ, as suggested by detection of Bves outside of the predicted domain (Fig. 3Bc, arrow). Taken together, both the in vitro and in vivo localization studies predict a role for Bves at the TJ.

Bves localizes early to points of cell-cell contact

Trafficking of proteins during early contact development has been crucial in establishing the function of adhesive proteins in epithelial sheet formation. If Bves is involved in establishing cell contacts and/or epithelial integrity, we postulate that Bves will behave like known junctional components (Adams et al., 1998b; Adams et al., 1996; McNeill et al., 1993; Vasioukhin et al., 2000). Thus, in an effort to determine how Bves is mobilized during epithelial maturation, we assessed how Bves protein localization changes during the formation, expansion and compaction of contacts, relative to the dynamic remodeling of the actin network. The EMC line was used for this study because it is a representative epithelial model of epicardial development where Bves was first identified. We determined that Bves is confined to an intracellular compartment in single cells (Fig. 4A, arrow) that colocalized with markers of the Golgi (Wada et al., 2001), while the actin network existed as a cortical ring at the cell periphery (Fig. 4A, arrowhead). Upon apposition of neighboring cells, Bves traffics to positions outside the cortical actin network (Fig. 4B, arrow), similar to the pattern observed with E-cadherin at forming contacts (Adams et al., 1998b; Ando-Akatsuka et al., 1999). As contacts expand, the cortical actin network collapses and increased Bves labeling is observed between apposing cells (Fig. 4C,D, arrows). Finally, as contacts compact and adhesions with additional cells are generated, Bves labeling is confined to regions of cell-cell contact and ultimately will encompass the entire cell circumference (Fig. 1A). Bves was never observed at the free surface of cells (Fig. 4, arrowheads). When placing Bves in the spatiotemporal framework of junction protein assembly, we found that the arrival of Bves at membrane protrusions is concurrent with E-cadherin, ZO-1 and β-catenin (supplementary material Fig. S1). These proteins localize to contacts at an early stage soon after an initial contact has developed, as indicated by actin and vinculin accumulation (Adams et al., 1998b; Ando-Akatsuka et al., 1999; McNeill et al., 1993; Rajasekaran et al., 1996; Vasioukhin et al., 2000). As shown in Fig. 2, Bves ultimately segregates with the TJ.
components ZO-1 and occludin as contacts mature. Thus, our findings demonstrate that Bves traffics to cell-cell contact points in a spatiotemporal manner appropriate for a protein involved in cell adhesion and/or maintenance of junctional complexes (Adams et al., 1996; Ando-Akatsuka et al., 1999; McNeill et al., 1993). While we report only this cell type, this pattern is representative for all epithelial cell types examined (data not shown).

Bves mimics ZO-1 response following challenges to epithelial integrity

Low Ca\textsuperscript{2+} and phorbol ester challenges to cell-cell adhesion and monolayer integrity have been used to explore junctional protein behavior and, in some cases, assign proteins to specific junctional complexes (Farshori and Kachar, 1999; Nishimura et al., 2002; Stevenson and Begg, 1994; Yamada et al., 2004). These culture manipulations were performed to elucidate Bves membrane properties more clearly.

Ca\textsuperscript{2+} switch assays challenge the integrity of an epithelial monolayer because many adhesion proteins such as cadherin rely on Ca\textsuperscript{2+} for their function. When Ca\textsuperscript{2+} is transiently depleted from a confluent, polarized monolayer of cells, the adhesive junctions disassemble and the cohesive nature of the epithelial sheet is compromised. This is represented by the loss of membrane-localized junctional proteins (Gumbiner et al., 1988; Nagafuchi et al., 1987). We found that EMCs can form a monolayer with primordial adhesive contacts following persistent culture in low Ca\textsuperscript{2+}, as has been observed with various other cells (Chaproniere and McKeehan, 1986; Ochieng et al., 1990; Shirakawa et al., 1986). Under these conditions, Bves was observed at contact points between the cortical actin networks of apposing cells (Fig. 5A, arrows) and was distributed around the cell circumference at confluence (Fig. 5B, arrows). This demonstrates that Bves localization and function at the membrane is Ca\textsuperscript{2+} independent, which correlates with our previous finding that exogenously expressed Bves induces L-cell aggregation in a Ca\textsuperscript{2+}-independent manner (Wada et al., 2001). Interestingly, ZO-1 mimicked this pattern at the cell surface after persistent Ca\textsuperscript{2+} depletion, and colocalized precisely with Bves (Fig. 5B, merge). Conversely, E-cadherin was absent from the cell membrane, as expected (Fig. 5C, middle panel arrow) and was only observed at the cell periphery in the presence of Ca\textsuperscript{2+} (Fig. 5C, middle panel insert).

While many studies have shown TJ assembly to be dependent on AJ assembly (Rothen-Rutishauser et al., 2002) and exogenous Ca\textsuperscript{2+} (Wilson, 1997), others have reported that ZO-1 is retained at the membrane of specific epithelial lines under various low Ca\textsuperscript{2+} culture manipulations (Fukuhara et al., 2002; Ide et al., 1999; Kartenbeck et al., 1991; Nishimura et al.,...
Bves modulates epithelial integrity

2002). Occludin was also absent from the cell membrane (data not shown) signifying that, although ZO-1 is retained at the membrane, intact TJ cannot form under these conditions. These findings reiterate the fact that Bves and ZO-1 are regulated differently than E-cadherin and the well-established Ca²⁺-dependent molecular network. These findings highlight the similarity of Bves and ZO-1 action under these conditions as well as underscore the Ca²⁺-independent nature of Bves function in epithelia. We demonstrate that Bves resides at the TJ with ZO-1 and occludin (Figs 2, 3). Here, we again find a striking similarity between Bves and ZO-1 response, further suggesting a role for Bves at the TJ.

The phorbol ester TPA activates protein kinase C, which regulates both tight junction biogenesis and Ca²⁺-induced polarization of epithelia (Balda et al., 1993; Stuart and Nigam, 1995). The addition of TPA to confluent epithelial cells following a Ca²⁺-switch is thought to bypass Ca²⁺-dependent cell-cell adhesion and initiate the formation of ‘TJ-like’ structures at the cell periphery (Balda et al., 1993; Farshori and Kachar, 1999; Nishimura et al., 2002; Ohsugi et al., 1997). Proteins such as JEAP and MAGI have been designated as TJ components because they localize to TPA-induced structures with ZO-1 and occludin (Ide et al., 1999; Nishimura et al., 2002). Therefore, we used this method to determine whether Bves would also aggregate at the membrane with known TJ components. This study was performed on MDCK (Fig. 6) and EMC (not shown) lines, which both produced similar results. In untreated cells, Bves, ZO-1, occludin and E-cadherin localize around the cell circumference (Fig. 6A,C,E). Following TPA treatment, Bves, occludin and ZO-1 are found in TPA-induced TJ-like structures at the cell membrane (B,D,F). E-cadherin labeling becomes diffuse and does not localize to these structures (F, arrows). Scale bar: 10 μm.

The Bves C-terminus interacts with the ZO-1 protein complex

To show that Bves is a component of an epithelial junction, it is crucial to provide evidence of physical association of Bves and known components. A GST pull-down assay was performed to determine whether Bves
binds, either directly or indirectly to components of the TJ. GST, GST N-terminal Bves and GST C-terminal Bves constructs were used to probe for interaction with candidate TJ proteins (Fig. 7A). ZO-1 was detected in the complex retained on beads bound with GST-C terminal Bves (Fig. 7B). Precipitated ZO-1 was not detected in the GST control fraction or, importantly, in the GST N-terminal Bves fraction, as this portion of the molecule has an extracellular distribution (Knight, 2003). However, an interaction between occludin and GST-Bves was not detected (Fig. 7C). This indicates that Bves may interact with the TJ directly or indirectly through the peripheral membrane protein ZO-1 and not membrane-bound occludin. This finding provides additional strength to the hypothesis that Bves is a functional component of the TJ, as these GST pull-down experiments demonstrate an association between Bves and the multimolecular complex containing ZO-1.

Knockdown of Bves function disrupts TJ integrity
To analyze Bves function, a method to eliminate and rescue Bves activity in an epithelial cell system was generated. We accomplished this by treating HCE cells with anti-human Bves MO and rescuing with transfected exogenous chicken Bves. HCE cells were used in this assay for several reasons. HCE parental cells are of human origin and display the same membrane distribution of Bves and TJ proteins (Fig. 8A) as observed in other cells, such as MDCK (Fig. 2). In addition, the cells more readily took up transfected DNA and MO than other lines tested, making HCE cells ideal for this study. Importantly, after examining numerous cell lines, HCE-R cells that stably express a ‘MO-rescuing’ chicken Bves construct appropriately trafficked the FLAG-tagged Bves to the membrane (Fig. 8B). This is highly significant, as this is the first cell culture system where expression and trafficking of exogenous Bves mirrors the endogenous pattern. Previously, we and others (Andree et al., 2000) have made several attempts to generate stable cell lines expressing Bves constructs and found that other cells did not properly traffic the Bves to the cell membrane upon transfection. However, this cell line affords, for the first time, an opportunity to identify Bves function and manipulate Bves in an in vitro environment. Furthermore, MO treatment of HCE cells results in a decrease of detectable membrane-localized Bves.

TER and localization of TJ markers provided readouts for epithelial integrity. Previously reported TER measurements for polarized HCE cells were typically between 200-800 Ω/cm² (Toropainen et al., 2001; Wang et al., 2004; Yi et al., 2000). Our culture conditions used in this study were designed to promote monolayer growth and the average measured resistance of the parental line was ~390 Ω/cm². As demonstrated in Fig. 8D, anti-Bves MO-treated HCE cells exhibit a significant loss of TER (asterisk, P<0.05) as compared with untreated or control MO-treated cells. HCE-R cells display nearly a 100% increase in TER over the parental control cell line. The TER of the HCE-R cell line overexpressing FLAG-tagged chicken Bves is only slightly decreased by anti-Bves MO treatment (Fig. 8D, double asterisk), indicating that exogenous Bves rescues the MO-induced drop in TER observed in the parental line. Immunolabeling of ZO-1 in MO-treated HCE cells demonstrates visually the compromise of epithelial integrity, which correlates with the TER data. The MO-induced phenotype is characterized by loss ZO-1 staining around the entire cell periphery and gaps in the epithelial sheet, while untreated and control MO cells show circumferential ZO-1 labeling (Fig. 8E). HCE-R cells exhibit retention of ZO-1 at the surface following anti-Bves MO treatment similar to controls (Fig. 8E, lower panel). As demonstrated by western blot analysis, MO treatment of HCE cells results in a decrease of Bves and ZO-1 (Fig. 8F) but transfection of chicken Bves into HCE cells results in an increase of Bves, as expected, as well as an increase of membrane-associated ZO-1. In addition, occludin, E-cadherin and β-catenin localization at the membrane is disrupted by MO treatment. This finding is not surprising, as reports from the literature suggest an interconnection of adhesive junctional complexes via protein-protein interactions (Ando-Akatsu et al., 1999; Itoh et al., 1993; Rothen-Rutishauser et al., 2002; Yamada et al., 2004; Yokoyama et al., 2001). For example, both TJ component ZO-1 and AJ protein β-catenin interact with α-catenin, which binds actin (Fanning et al., 1998; Imamura et al., 1999). Thus, it is highly likely that disruption of the TJ will result in a
Bves modulates epithelial integrity

Discussion

The current study was initiated to investigate the putative involvement of Bves in cell adhesion and to test the hypothesis that Bves functions endogenously to regulate epithelial integrity. Given the broad distribution of Bves in a variety of epithelial types, the present data predict a conserved, significant and wide-ranging function of this novel gene family. In addition, the dynamic distribution pattern during contact formation correlates with a proposed involvement in cell-cell adhesion. Immunolocalization and physiological challenge

Fig. 8. Compromise of TJ integrity following knockdown and rescue of Bves in HCE cells. (A) Expression of Bves and ZO-1 at the cell periphery in parental HCE cells. (B) HCE-R cells stably expressing the ‘rescue’ chicken Bves-FLAG construct exhibit labeling of the membrane by both FLAG and Bves antibodies. Note the intracellular accumulation of transfected chicken Bves. (C) Treatment with anti-human Bves MO results in a loss of Bves at the cell periphery, as compared to untreated (NT) and control MO. (D) TER measurements were performed on HCE and HCE-R. Parental HCE cells exhibited a TER value of approximately 390 Ω/cm². Treatment with anti-human Bves morpholino resulted in a 40% decrease (P<0.05) in TER (asterisk), while control MO did not significantly alter the TER. The TER of HCE-R cells doubled to 775 Ω/cm² and, following anti-human Bves MO (double asterisk), a drop of only 20% (P<0.05) in TER was observed. (E) ZO-1 immunofluorescence performed on Transwell inserts following TER measurements. Parental HCE cells and HCE-R cells were treated with control and anti-human Bves MO. Bves and ZO-1 expression is disrupted in HCE cells treated with anti-human Bves MO (top right panel). Morpholino treatment does not alter the expression pattern of ZO-1 in HCE-R cells (lower right panel). (F) Western blotting demonstrates that membrane ZO-1 and Bves increase with exogenous expression of Bves in HCE-R cells, but decrease following treatment with Bves-MO, as compared with the parental HCE line. (G) Membrane integrity of junction proteins occludin, E-cadherin and β-catenin is disrupted following Bves MO treatment. Scale bars: 10 μm.
studies suggest Bves resides at or near the TJ, a multi-protein complex important for epithelial cell function. Thus, Bves is spatiotemporally regulated in a manner necessary to fulfill requirements of a junction/adhesion protein. GST pull-down assays demonstrate a physical link to the complex containing an established TJ component, ZO-1. Furthermore, MO knockdown and rescue experiments reveal that Bves is necessary for the stability of an epithelial monolayer in vitro. Taken together, our data establish that Bves exhibits a functional role in the maintenance of epithelial integrity and we are the first to show that Bves could be an important molecular element at the tight junction.

Significant to this study is the placement of Bves into a definitive junction within the terminal bar of epithelial cells. While previous findings determined that Bves localizes to the cell membrane and confers adhesion to L-cells after transfection, the protein had not been assigned to a subcellular domain prior to this report. Our current immunohistochemical, confocal and immuno-EM analyses of cell lines and the gastric epithelium indicate that Bves co-localizes with occludin and ZO-1. Also, Bves responds like both TJ markers following TPA challenge, an assay used to identify components of the TJ, such as JEAP and MAGI/BAP1 (Ide et al., 1999; Nishimura et al., 2002). Importantly, of these two TJ proteins, only the ZO-1-containing complex was shown to interact with Bves. We postulate that we detect an interaction with ZO-1, and not occludin, because of the relative strengths of interaction between Bves and these proteins. While Bves interaction with ZO-1 may be direct or indirect, through a third protein, it appears to be tightly associated with ZO-1. Although we believe that the ZO-1/Bves interaction occurs at the membrane, it is not out of question that the association we detected occurs elsewhere in the cell.

Although ZO-1 and occludin are both TJ components, ZO-1 clearly has roles outside the TJ, and its behavior is often distinct from occludin (Fanning et al., 1998; Gottardi et al., 1996; Itoh et al., 1997; Schneeberger and Lynch, 2004). ZO-1 is a scaffolding protein that interacts with occludin, claudins and ZO proteins at the TJ in epithelial cells but also possesses an increasing number of other binding partners (Balda and Matter, 2000; Gonzalez-Mariscal et al., 2003; Matter and Balda, 2003). For example, ZO-1 can be detected at contact points with E-cadherin and catenin proteins, well before occludin, claudins and other TJ components translocate to the cell periphery (Ando-Akatsuka et al., 1999). Also, in non-epithelial cells where TJ are not formed and occludin is not expressed, ZO-1 functions as a crosslinker between the cadherin complex and actin network, through interaction with AJ protein α-catenin (Itoh et al., 1993) and the nectin-afadin complex (Yokoyama et al., 2001). In the present study, we found that Bves exhibits greater similarity to ZO-1 in several cases. For example, Bves localizes to cell-cell contacts with the cadherin/catenin complex, mimicking what is observed with ZO-1 during contact formation (Ando-Akatsuka et al., 1999). Interestingly, persistent culture of EMCs in low Ca2+ revealed a remarkable parallel between the responses of Bves and ZO-1, even when E-cadherin and occludin failed to localize to the membrane. Also, Bves localizes with ZO-1 in some non-epithelial cells lacking functional TJs and expression of occludin. Finally, Bves was detected outside the predicted TJ domain in ultrastructural studies. Thus, while we establish that Bves is concentrated at the TJ, we find that Bves exhibits membrane properties like ZO-1 rather than occludin. With the finding that Bves interacts with the ZO-1-containing complex, our data suggests that while Bves clearly has a role at the TJ, the possibility remains that Bves may have roles expanding beyond the TJ-like ZO-1. We currently have no definitive explanation for this, although it is likely that this similarity of ZO-1 and Bves can be correlated with a common function.

To further investigate Bves function in epithelia, we established the SV40-t HCE cell model system where Bves function can be disabled following morpholino treatment and rescued with exogenous protein expression. We combined these MO experiments with TER analysis, a method used to confirm a functional role for proteins at the TJ (Cereijido et al., 1978; Gonzalez-Mariscal et al., 2003; Gumbiner and Simons, 1986; Sonoda et al., 1999). Recently, the proteins coxsackie- and adenovirus receptor-like membrane protein (CLMP) and myosin light chain kinase (MLCK) have been established as TJ components using this assay (Clayburgh et al., 2004; Rascherperger et al., 2004). Bves knockdown by MO in HCE cells results in the rapid loss of TER, epithelial polarization and the disassembly of cell junctions. Similarly, RNAi suppression of junctional adhesion molecule 1 (JAM-1) or partitioning-defective 3 (PAR-3) disrupted TJ integrity, caused a mislocalization of related proteins and caused a drop in TER values (Chen and Macara, 2005; Mandell et al., 2005). In addition, Chen et al. rescued the alterations by expression of human PAR-3 (Chen and Macara, 2005). It is important to note that the transfection of chicken Bves into HCE cells not only rescues the MO knockdown effects as demonstrated by retention of ZO-1 at the membrane, but drastically increases the TER of both control and MO-treated HCE-R cells. This signifies that Bves could be required for integrity of the epithelial sheet by its influence at the TJ. We postulate that the overexpression of Bves strengthens the TJ seal, thus generating a significantly higher TER value, as has been shown with overexpression of other TJ proteins (Cohen et al., 2001; McCarthy et al., 1996; Rascherperger et al., 2004). Furthermore, this increase in TER and rescue demonstrates that the observed phenotype is Bves-dependent and suggests a conservation of Bves function between species. Taken together, the present study is the first to assign a function for Bves in epithelial maintenance and integrity and establishes that Bves may be an important molecular component of the TJ.

We interpret our findings as supporting localization and function for Bves at the TJ. However, as discussed, we predict that Bves can act outside the TJ as well, since it localizes with ZO-1 in some non-epithelial cells. Throughout the literature, studies show that proteins found at the TJ can have cellular functions ranging from polarity establishment (Roh et al., 2002) to roles in immune system regulation (Coyne et al., 2004). Reports in recent years have placed an increasing number of proteins at the TJ, many of which are known as MAGUK proteins because they contain PDZ, SH3, and GUK domains. MAGUK proteins include the ZO family members, proteins of the PAR/PATJ/Clumbs, MAGI and MUPP groups (Gonzalez-Mariscal et al., 2003). In addition, other ‘non-traditional’ molecules lacking these domains are also recruited to the TJ and co-localize with ZO-1 and function as adaptor proteins, regulatory proteins, or transcriptional regulators (Matter and Balda, 2003; Schneeberger and Lynch, 2004). Not
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