Novel membrane protein shrew-1 targets to cadherin-mediated junctions in polarised epithelial cells

Sanita Bharti¹, Heike Handrow-Metzmacher¹, Silvia Zickenheiner¹, Andreas Zeitvogel¹, Rudolf Baumann² and Anna Starzinski-Powitz¹,*

¹Humangenetik fuer Biologen, Johann Wolfgang Goethe-Universität, Siesmayerstr. 70, D-60054 Frankfurt, Germany

Address of corresponding author:
Humangenetik fuer Biologen, Johann Wolfgang Goethe-Universität, Siesmayerstr. 70, D-60054 Frankfurt, Germany
Tel. 49-69-79824769; Fax: 49-69-79824732
E-mail: starzinski-powitz@em.uni-frankfurt.de

²Bürgerhospital Frankfurt, Section of Gynecological Endocrinology and Fertility Surgery, Nibelungenallee 37-41, D-60318 Frankfurt, Germany
ABSTRACT

While searching for potential candidate molecules relevant for the pathogenesis of endometriosis, we discovered a 2910 bp cDNA encoding a novel putative 411 amino acid integral membrane protein which we called shrew-1. The putative open-reading frame was confirmed with antibodies against shrew-1 peptides which labelled a protein of ca. 48 kDa in extracts of shrew-1 mRNA positive tissue and also detected ectopically expressed shrew-1. Expression of epitope-tagged shrew-1 in epithelial cells and analysis by surface biotinylation and immunoblots demonstrated that shrew-1 is indeed a transmembrane protein. Shrew-1 is able to target to E-cadherin-mediated adherens junctions and interact with the E-cadherin-catenin complex in polarised MCF7 and MDCK cells, but not with the N-cadherin-catenin complex in non-polarised epithelial cells. Direct interaction of shrew-1 with β-catenin in in vitro pulldown assay suggests that β-catenin might be one of the proteins that targets and/or retains shrew-1 in the adherens junctions. Interestingly, shrew-1 was partially translocated in response to scatter factor (ligand of receptor tyrosine kinase c-met) from the plasma membrane to the cytoplasm where it still colocalised with endogenous E-cadherin. In summary, we introduce shrew-1 as a novel component of adherens junctions, interacting with E-cadherin-β-catenin complexes in polarised epithelial cells.

Keywords: E-cadherin/N-cadherin/c-met/scatter factor/non-polarised cells.
INTRODUCTION

Epithelial cell morphology and tissue architecture depends on cell polarity which is defined by the apical side of the cell facing the lumen, the basal side, and the lateral part with different types of junctions (adherens junctions, tight junctions, desmosomes and gap junctions (Farquhar and Palade, 1965; Burridge et al., 1988; Chrzanowska-Wodnicka and Burridge, 1996; Gumbiner, 1996). Central to the maintenance of epithelial cell polarity are the adherens junctions (zonula adherens) for which cell-cell adhesion is a coordinating force. Adherens junctions are known to play key roles in forming and maintaining tight junctions and desmosomes, in addition to concentrating many biologically active molecules such as membrane receptors, signalling molecules and oncoproteins (Tsukita et al., 1992; Aberle et al., 1996; Anastasiadis et al., 2000; Gumbiner, 2000; Nagafuchi, 2001). An understanding of the processes and proteins leading to the formation of cell-cell junctions as well as controlling their dynamics can provide a clue to the development and function of epithelia in both physiology and pathology (Eaton and Simons, 1995; Pollack et al., 1998).

The formation of epithelial adherens junctions is mediated by the calcium-dependent cell adhesion protein E-cadherin, a so-called classical cadherin. The polarising function of E-cadherin depends largely on its clustering and interaction with the actin-based cytoskeleton through a number of cytoplasmic components (Gumbiner, 1996; Takeichi, 1995). Important cytoplasmic scaffolding proteins of the E-cadherin complex are in particular β-catenin, α-catenin and proteins of the p120(ctn) family, but also AF-6 (Boettner et al.,
2000), ponsin (Mandai et al., 1999), erbin (Borg et al., 2000) and conceivably others. The direct or indirect interaction of these proteins with the cytoplasmic part of E-cadherin is important for the stabilization of the complexes at the adherens junctions. Crystallographic studies have revealed that the cadherin cytoplasmic domain is essentially disordered in the absence of binding partners (Huber et al., 2001). Moreover, transmembrane proteins such as nectin (Tachibana et al., 2000) and vezatin (Küssel-Andermann et al., 2000) are also found in the adherens junctions to which they are recruited, directly or indirectly, via α-catenin (Nagafuchi, 2001) and thus contribute to the interaction of E-cadherin with the cytoskeleton.

Adherens junctions are highly dynamic structures that can undergo rapid but nevertheless regulated assembly and disassembly, as in epithelial-mesenchymal transitions. Such processes are needed, for example, when cells decrease the strength of their adhesion in order to move. If deregulated in pathological situations these phenomena may participate in establishing invasion and metastasis of carcinomas, as has been shown in many cases (Shimoyama and Hirohashi, 1991; Shiozaki and Mori, 1991; Takeichi, 1991). The dynamics of adherens junctions are influenced by regulatory proteins such as kinases for example c-src, c-Fyn or phosphatases known to be present in adherens junctions. Regulatory signals also include intracellular proteins such as Ras, Rac and Cdc small GTPases or the EGF/EGF receptor signalling system (Gumbiner, 1996; Gumbiner, 2000). Another particular example is the receptor tyrosine kinase c-met with the ligand scatter factor (SF; also known as
hepatocyte growth factor HGF). Stimulation of epithelial cells with SF/HGF induces disassembly of the adherens junctions, whereby cells loose their typical morphology and become motile and invasive (Birchmeier et al., 1993; Watabe et al., 1993; Weidner et al., 1996). Along with the disassembly of epithelial cells, E-cadherin-catenin complexes may be endocytosed and degraded or recycled, quite likely in caveolin-containing vesicles (Akhtar and Hotchin, 2001). Endocytosis and degradation of E-cadherin can be controlled by HAKAI, recently identified as a novel ubiquitin E3 ligase interacting with E-cadherin, which can modulate cell adhesion and increases the response of epithelia to scatter factor (Fujita et al., 2002).

In this paper we introduce and characterize a novel membrane protein, shrew-1, that targets to epithelial adherens junctions and interacts with cadherin-catenin complexes in polarised but not in non-polarised cells.
RESULTS

Identification of shrew-1 from endometriotic cell line
A cell line (EEC145T) from endometriosis lesions which has recently been established in our laboratory was found to be epithelial in nature (cytokeratin positive, E-cadherin negative) (Zeitvogel et al., 2001). The fact that this cell line became non-invasive after a few passages prompted us to use it as a tool for identifying markers differentially expressed during endometriosis (Figure 1A). We therefore performed Differential Display Reverse Transcriptase PCR (DDRT-PCR) with the invasive (p17) and non-invasive (p33) passages of this cell line. This reproducibly resulted in the isolation of a 391 bp DDRT-PCR fragment that was differentially expressed in the invasive EEC145T cell line. Northern blots (Figure 1B) using the 391 bp fragment as a probe confirmed the presence of a corresponding message in invasive EEC145T cells and revealed an mRNA of approximately 4 kb. The gene and its products (mRNA and protein) were called shrew-1.

Isolation of the cDNA and nucleotide sequence analysis
Database searches for sequences identical to shrew-1 DDRT cDNA sequences revealed ESTs with identical sequences from various parts of the brain, kidney and foetus, as well as colon carcinomas. Screening of a ZAP Express™/EcoRI/XhoI custom cDNA phage library constructed from RNA of invasive passage p17 of EEC145T led to the isolation of phagemid clone Q2A containing an insert of 2204 nucleotides including the original DDRT-PCR fragment. Longer cDNA fragments could not be obtained from this library. Therefore, we isolated the rest of the cDNA by 5’ and 3’ RACE experiments using commercially available Marathon-Ready™ cDNA from human brain, which is also positive for shrew-1. The cDNA finally obtained
contained 2910 nucleotides and was identical to mRNA sequences in the EEC145T cells as revealed by overlapping RT-PCRs and DNA sequencing (accession number of shrew-1:AY282806). It encodes a putative protein of 411 amino acids, sharing more than 90% identity with a putative mouse counterpart derived from ESTs published in the database (accession number BI990953). The amino acid composition (Figure 2A) of shrew-1 predicts a highly alkaline protein with an isoelectric point of 9.86 and a theoretical molecular mass of 44.5 kDa. A computer search for conserved protein motifs revealed a putative signal peptide of approximately 43 aa (bold in Figure 2A), a putative transmembrane domain (underlined in Figure 2A) and some potential sites for phosphorylation, glycosylation and myristylation (not shown).

It should be noted that the nucleotide sequence of a cDNA isolated from a fibrosarcoma (accession number AF175409; but not described otherwise) allows the generation of an ORF which is completely identical to that of shrew-1. So far, there is no evidence that shrew-1 is a member of any protein family because there are no conserved domains in other proteins, either in human or in any other organism including yeast, Drosophila or C. elegans. Orthologous protein sequences of shrew-1 itself were found in mouse and zebrafish but not in Drosophila, C.elegans or yeast suggesting that shrew-1 might be a protein which is restricted to vertebrates.

Expression of shrew-1 protein

Two different types of antibodies were generated to analyse expression at the protein level. First, custom-made mouse monoclonal antibodies were produced against a synthetic peptide deduced from the putative extracellular domain (for sequence see Material and Methods). The resulting monoclonal antibodies were tested against
protein extracts from human pancreas and uterus in an immunoblot, since both these tissues were found to contain shrew-1 mRNA as shown by Northern blot and RT-PCR analysis (data not shown). As shown in Figure 2B, lanes 1 and 2, both tissues were found to contain a protein of approximately 48 kDa corresponding to the predicted size of the shrew-1 protein.

Secondly, polyclonal antibodies were generated in rats by genetic immunization against the putative cytoplasmic domain of shrew-1 (in collaboration with Genovac, Freiburg, Germany). This antibody also recognised endogenous protein from tissue extract (described below).

For ectopic expression, shrew-1 was cloned into two different expression vectors fused to either a 10 aa long birch profilin tag (shrew-1-BP) or a green fluorescent protein tag (shrew-1-GFP). To check whether these vectors expressed the predicted open reading frame protein of 411 aa we performed different kinds of experiments. First, shrew-1-BP was translated radioactively in vitro using a reticulocyte lysate kit (see Material and Methods). SDS-PAGE and autoradiography revealed that shrew-1 cDNA encoded indeed a protein of approximately 48 kDa (Figure 2B, lane 4). The positive control used for in vitro translation was luciferase cDNA supplied by the manufacturer (Figure 2B, lane 3). In extracts of human epithelial MCF7 cells ectopically expressing shrew-1-GFP, anti-GFP antibody detected a protein of the expected size of approximately 75 kDa in Western blots (Figure 2B, lane 5). Shrew-1 rat polyclonal antibody against the putative cytoplasmic polypeptide sequence gave a signal of comparable size in these extracts (Figure 2B, lane 6). The shrew-1 monoclonal antibodies that detected shrew-1 in pancreas and uterus cell extracts (Figure 2B, lanes 1 and 2) also detected the recombinant transfected shrew-1-GFP in
MCF7 cell extracts (Figure 2B, lane 7). It should be noted that, although MCF7 cells do contain shrew-1 mRNA endogenous protein could not be detected possibly due to low abundance (data not shown).

In order to indicate unambiguously the authenticity of the endogenous protein band as shrew-1 protein, immunoprecipitation (IP) experiment was performed with uterus extract using polyclonal rat antibody and subsequent immunoblot (IB) with the monoclonal antibody (Figure 2C, lane 2 and 3). Vice-versa, IP was performed with the monoclonal antibody and IB with the polyclonal antibody (Figure 2D, lane 2 and 3). Both approaches revealed a protein of the expected size as in the input cell extracts (10% of the total cell extract). The fact that the two antibodies against peptides from two different regions in shrew-1 sequence detected apparently the same protein additionally confirmed that the predicted shrew-1 open reading frame is endogenously expressed in mammalian cells.

**Membrane localization and orientation of shrew-1**

Shrew-1 fused to two different tags (to rule out the possibility that cellular localization is affected by the tags) was used to determine the cellular localization of the protein. These studies were performed in epithelial cell lines 12Z, RT112, EJ28 and MCF7 transiently transfected with shrew-1-GFP and shrew-1-BP. In all cases, major pools of shrew-1 appeared to be localized at the plasma membrane, especially at the regions of cell-cell contacts, irrespective of whether RT-PCR (data not shown) showed that the cell lines contained endogenous shrew-1, namely MCF7 and 12Z (Figure 3; A, D, E, H) or not, i.e. RT112 and EJ28 (Figure 3; B, C, F, G). The cells shown in the pictograph are mainly shrew-1 transfected cells with a few neighboring cells.
In order to check whether shrew-1 is exposed on the cell surface, shrew-1-GFP was transiently transfected into MCF7 cells. Surface-exposed proteins were then biotinylated using a membrane-impermeable biotin. After cell lysis and detergent solubilization, biotinylated proteins were isolated by incubation with agarose-coupled neutravidin beads. Immunoblotting using antibodies against GFP revealed that shrew-1-GFP was present in the biotinylated protein fraction (Fig. 4A, lane 4) confirming that shrew-1 is an integral component of the plasma membrane. E-cadherin, a transmembrane protein (Figure 4B, lane 3) and pyruvate kinase, a cytosolic protein (Figure 4C, lane 2) were used as positive and negative controls, respectively.

Furthermore, we tested whether the carboxyl terminus of shrew-1 is cytoplasmic by performing permeabilization studies. MCF7 cells were transiently transfected with shrew-1 tagged with a C-terminal GFP tag (shrew-1-GFP). One aliquot of the transfected cells was permeabilized (Figure 5; A, B) and immunodetection was performed using GFP antibody (Figure 5; B, D) whereas the other aliquot was not permeabilized (Figure 5; C, D) and immunostaining was performed on live cells using GFP antibody in the presence of sodium azide to prevent antibody-induced capping. The autofluorescence from shrew-1-GFP (Figure 5; A, C) could be seen in both cases, but antibody staining could only be seen with cells that were permeabilized. This clearly implies that the C-terminus is indeed cytoplasmic. A comparable result was obtained when a similar experiment was performed in MDCK cells (data not shown).
Colocalization of shrew-1 with E-cadherin at the adherens junctions

As seen in Figure 3, shrew-1-GFP appeared to be concentrated mainly at sites of cell-cell contact. This was even more evident in epithelial cells that expressed E-cadherin at the membrane such as MCF7 (Figure 3 and 6) and MDCK cells (see also Figure 6). We therefore asked whether shrew-1 and E-cadherin colocalise in these cells. Shrew-1-GFP was transfected into MCF7 and MDCK cells and subsequently costained for endogenous E-cadherin by indirect immunofluorescence. Optical sectioning with confocal microscopy revealed that E-cadherin colocalises with shrew-1-GFP along the xy-axis (Figure 6; A-D). Additionally, when the sections were recorded along the xz-axis (Figure 6; E and F), shrew-1 was found to colocalise with E-cadherin at the junctions.

Since E-cadherin is a marker of adherens junctions, we presume that shrew-1 is also present in these junctions. Whether this colocalization was the result of shrew-1 interacting specifically with E-cadherin or just a coincidence was further investigated.

Interaction of shrew-1 with cadherin-β-catenin complexes in polarised and non-polarised cells

To check whether shrew-1 can complex with E-cadherin, MCF7 cells were transiently transfected with shrew-1-GFP or the vector alone and grown to confluency. Cell extracts were prepared and transfection efficiencies were monitored by immunoblotting (IB) 10% of the total cell extract using GFP antibody (Figure 7A; Input). The remaining cell extract was immunoprecipitated (IP) with GFP antibody and protein G-sepharose beads, then the whole complex was immunoblotted using E-cadherin antibody (Figure 7A, lanes 3, 4). E-cadherin could be detected in the immunocomplex pulled down by monoclonal anti-GFP antibody. Complexing of
shrew-1 and E-cadherin could be observed in confluent but not in subconfluent cells where, however, colocalisation of endogenous E-cadherin and shrew-1 could already be seen (for the latter see also Figure 8). This suggested that shrew-1 complexes with cadherin-catenin protein detectably only upon the formation of junctions (see Discussion). β-catenin was also detected on reprobing the same blot with the beta-catenin antibody (Figure 7A). The reverse experiments confirmed the same results when IP was done with E-cadherin antibody and shrew-1-GFP could be detected in the same complex (Figure 7B).

Furthermore, we analysed the ability of shrew-1 to interact with cadherins in epithelial cell lines that are unable to form adherens junctions (for example EJ28 cells, an invasive human bladder carcinoma cell line expressing N-cadherin; Figure 7C). EJ28 cells were transfected with shrew-1-GFP and GFP alone. Monoclonal anti-GFP antibody was used for Co-IP assays performed as described above. For IB detection we used anti-N-cadherin, anti-β-catenin and anti-GFP antibodies. N-cadherin and β-catenin could not be detected in the immunocomplex pulled down by anti-GFP antibody. These data reiterate that shrew-1 can interact with cadherin-catenin complexes in junctions of polarised epithelial cells but not with cadherin-catenin complexes (here N-cadherin) in non-polarised cells.

The results shown so far do not indicate whether the interaction between E-cadherin and shrew-1 is due to direct binding of the proteins or is caused by an intermediate protein such as a scaffolding protein in the complex (β-catenin being a candidate). We therefore performed in vitro pull-down assays (see Material and Methods) between the cytoplasmic domain (CPD) of shrew-1 (used as GST fusion protein) and in vitro translated β-catenin (Figure 7D, lane 1) or full-length E-cadherin (not shown).
While E-cadherin could not be pulled down by GST-CPD-shrew-1 (data not shown), β-catenin clearly interacted with the cytoplasmic domain of shrew-1 (Figure 7D, lane 4).

Taken together, these data support the idea that shrew-1 interacts with β-catenin in epithelial adherens junctions. However, this does not exclude that shrew-1 binds to other as yet unidentified components of the adherens junctions.

**Effect of addition of SF/HGF on colocalization of shrew-1 and E-cadherin**

We decided to disrupt cellular junctions by adding scatter factor/hepatocyte growth factor (SF/HGF) to find out whether shrew-1 and E-cadherin still colocalise after junction disruption. SF/HGF is a cytokine that acts as a morphogen leading to epithelial-mesenchymal transitions. It is known to disrupt E-cadherin mediated junctions in MDCK cells through activation of its receptor c-met. During disruption, E-cadherin is transiently transported into recycling vesicles reported to contain caveolin-1 (Akhtar and Hotchin, 2001).

MDCK cells transiently transfected with shrew-1-GFP were seeded at very low density on cover slips and were grown till the formation of islands. SF/HGF was added at a concentration of 20 ng/ml and the effect was monitored on cover slips from the same culture dish at 0h, 4h, 8h and 15h (Figure 8). After 8h a drastic change in the intracellular distribution of shrew-1 was observed. GFP fluorescence at the plasma membrane was reduced and intracellular particulate structures were labelled, which also stained for E-cadherin (marked with arrow heads). When the scattering effect of SF/HGF was gone after fifteen hours, GFP fluorescence and E-cadherin were again prominent at the plasma membrane. These results suggest that
upon disruption of junctions by a physiological stimulus shrew-1 is translocated together with E-cadherin to intracellular vesicles. Although it seems from these experiments that shrew-1 and E-cadherin have a comparable pattern of internalization, it can however not be discriminated whether this is caused by a weak interaction not detectable by coimmunoprecipitation or simply by a similar response to stimulation by SF/HGF.

DISCUSSION

Shrew-1 in junctional and non-junctional protein complexes

Here, we described the isolation and characterization of a novel protein, shrew-1 that is able to target to E-cadherin-mediated adherens junctions. Confocal microscopic analysis of transiently expressed shrew-1 revealed its localization at the lateral part of the cell membrane, where it colocalised and coimmunoprecipitated with endogenous E-cadherin, also present at the lateral part of the membrane in polarised MDCK cells (Le Bivic et al., 1990; Shore and Nelson, 1991). It has been shown in many studies that adhesion is mediated by the cytoplasmic domain of E-cadherin linking to the actin cytoskeleton (Takeichi et al., 1988) via associated proteins such as β-catenin, and α-catenin (Aberle et al., 1994). Interestingly, direct interaction between β-catenin and shrew-1 in an in vitro pull-down assay suggested that shrew-1 might be linked to the E-cadherin-mediated junctional complex via β-catenin.

The fact that no interaction could be found between shrew-1 and N-cadherin-β-catenin complex in non-polarised cells however favours the idea that β-catenin alone is not sufficient to target shrew-1 into adherens junctions, but other, as yet unidentified components might also be necessary. One component could be E-cadherin itself, although we were unable to identify a direct interaction between full-
length E-cadherin and the cytoplasmic domain of shrew-1 in vitro. It is still possible that an interaction between E-cadherin and shrew-1 might be mediated via the transmembrane and/or the extracellular domains.

This interpretation also raises the possibility that shrew-1 is in fact targeted to specific cell contact sites, e.g. adherens junctions, rather than merely binding to E-cadherin per se. In line with such an idea is the observation that coimmunoprecipitation of shrew-1 with the E-cadherin-catenin complex was dependent on the formation of junctions and, in spite of colocalisation in immunofluorescence between shrew-1-GFP and endogenous E-cadherin, complexing could not be observed in subconfluent cells.

An explanation for this could be that the interaction is rather weak at this stage of cell growth and only stabilises when cells junctions mature in confluent cells. The second explanation could be that initially E-cadherin and shrew-1 are in two independent complexes which only interact in rather mature junctions. This might be a sort of anchoring requiring additional bridging protein(s).

The colocalization of shrew-1 with E-cadherin observed at the cellular junctions in MDCK cells transiently expressing shrew-1 also persisted when the junctions were disrupted by the physiological stimulus SF/HGF and during subsequent endocytosis of E-cadherin. It can however not be discriminated whether this is caused by a weak interaction not detectable by coimmunoprecipitation or simply by a similar response to stimulation by SF/HGF.
The vesicles in which E-cadherin and shrew-1 were endocytosed are found to contain caveolin-1 (data not shown), an integral membrane protein of caveolae, which is in agreement with published reports with regard to E-cadherin (Akhtar and Hotchin, 2001). It remains to be tested whether this colocalization during endocytosis is of physiological relevance particularly with regard to shrew-1 positive structures.

**Shrew-1 protein sequence and potential functional features**

The deduced protein sequence of shrew-1 exhibits a number of unusual features. Computational analysis predicted a transmembrane domain and an unusual signal peptide. The functional integrity of these domains was implied by our experiments showing that the shrew-1 cDNA encodes a membrane-spanning protein with a cytoplasmic C-terminus.

Surprisingly, no other structural features such as an $\alpha$-helix or immunoglobulin-like domains could be predicted for the protein. Instead, we identified so-called low complexity regions (Peer Bork and Gisbert Schneider, personal communications) for which no structure can be predicted with the algorithms available (Schultz et al., 1998). One general idea about the functional relevance of these low complexity regions is that they only acquire a rigid structure (e.g. an $\alpha$-helix) upon interaction with another protein partner or oligomerisation into a protein complex (Wright and Dyson, 1999). This has been reported for SNAREs, which play a role in vesicle docking and fusion, and which only form an $\alpha$-helix when they bind to their interaction partner (Fasshauer et al., 1997; Jahn and Sudhof, 1999).

Further analysis of the shrew-1 protein sequence revealed a putative nuclear localization signal (NLS) in the predicted extracellular domain and putative
glycosylation signals in the cytoplasmic domain (unpublished observations). Although these signals might be some artefacts of computational analysis, a functional relevance of these findings cannot be excluded. For example, it has recently been shown that the EGF receptor, an integral plasma membrane protein, can travel to the nucleus where it apparently exhibits a specific function possibly in the regulation of gene activities (Waugh and Hsuan, 2001).

In conclusion, we isolated a novel integral membrane protein, which might play a role in the function and/or regulation of E-cadherin-mediated junctional complexes. As an integral component of the adherens junctions and as a protein complexing with E-cadherin-β-catenin complex, investigation of shrew-1 gene activity and function could be important to improve our understanding of the dynamics of adherens junctions in both physiological and pathological processes i.e. tumour progression and metastasis.

MATERIAL AND METHODS

DDRT-PCR and RACE

DDRT-PCR was essentially performed as described (Liang and Pardee, 1992) using a commercially available kit (Genhunter Corporation, Nashville, USA). Briefly, the Genhunter kit contains four different downstream primers (T12MA, T12MG, T12MT, T12MC) used for first strand cDNA synthesis and twenty different upstream primers (AP-1 to AP-20) for amplification. The cDNAs amplified from poly A+ RNA from either invasive or non-invasive EEC145T cells in the presence of radioactively labelled nucleotides were separated on polyacrylamide gels, autoradiographed and the band patterns compared. Amplification products differentially and reproducibly found in
either of the EEC145T variants (invasive or non-invasive) were cut out of the gel, re-amplified and cloned into a vector. The nucleotide sequences of the cloned products were determined and the differential expression pattern of the identified sequences validated by RT-PCR and Northern blots. The kit for RACE (Clontech, Germany) was used according to the manufacturer’s instructions. Briefly, PCR was performed using Marathon ready cDNA and the anchor primer AP1 provided, which annealed specifically to the linker sequence on the cDNA. The sequence of the gene specific primer from within the known 391 bp sequence used for 3´RACE was 5´-gtgttggaagatgctacc-3´ and that of the primer used for 5´RACE was 5´-tgaactcagtctctgtgg-3´. To confirm the specificity of the product nested RACE was performed with a nested gene specific primer for 5´RACE: 5´-ggatttggcagcagctgg-3´ and a nested primer provided with the kit for 3´RACE: 5´-tagacgttggtcttgaggg-3´. The product was cloned into a vector and sent for sequencing.

Phage library
The ZAP Express / EcoRI / XhoI custom cDNA phagebank was constructed by Stratagene from the poly A(+) mRNA from passage 17 of EEC145T. Screening was performed according to standard protocols (Short et al., 1988).

Plasmid constructs and protein expression
Shrew-1 cDNA isolated from the epithelial endometriotic cell line EEC145T was cloned into eukaryotic expression vectors pEGFP-N3 (Clontech, Heidelberg, Germany) and into pcDNA3.1(+) with a BP tag (Kaufmann et al., 2000) using restriction sites introduced by PCR. PCRs were performed using Platinum Pfx-DNA polymerase (Invitrogen, Karlsruhe, Germany). The primers used for cloning into pEGFP-N3 contained the restriction sites BglIII and Acc651. The sequence of the
forward primer was: 5´-agatctgaccatgtgga ttcaacagc-3´ and the reverse primer: 5´-
ggtaccgcaggagatttcaacc-3′. For cloning into the pcDNA 3.1(+) vector, the restriction
sites HindIII and EcoRI were incorporated using the forward primer: 5´-
aagcttgaccatgtggattcaacagc-3´ and the reverse primer: 5´-
gaattccagcaggagatttcaacc -3´.

β-catenin cloned into pcDNA vector was a kind gift from the group of R. Kemler (MPI
for Immunology, Freiburg, Germany). The cytoplasmic domain of shrew-1 was cloned
using PCR amplification into the pGEX-5X1 vector (GST-CPD-shrew) using primers
with an EcoRI site incorporated into the forward primer and SalI in the reverse primer.
The forward primer had the sequence: 5´-atcgaattcatgtctgggggcaatac-3´ and the
reverse primer: 5´-ggctcgagtcgacttatatttctttc tg-3´. The protein was expressed in
bacteria and purified as described before (Kaufmann et al., 2000).

Antibodies
Monoclonal anti-GFP antibody was obtained from Clontech (Heidelberg, Germany).
Monoclonal pyruvate kinase antibody was obtained form Schebotech (Wettenberg,
Germany). Monoclonal E-cadherin (clone 36) and monoclonal beta-catenin (clone 14)
were obtained from Transduction Laboratories, Pharmingen, Los Angeles, USA.
Another monoclonal antibody against E-cadherin, 5H9 and a Pan-cadherin were
purchased from Sigma, Deisenhofen, Germany. Monoclonal N-cadherin antibody
(Clone 3B9) was purchased from Zymed, California, USA. DECMA-1, a rat
monoclonal antibody against E-cadherin was a kind gift from R. Kemler.
Monoclonal antibodies were generated against shrew-1 in mice using the peptide sequence \( \text{NH2-ACMTLQTKGFTESLDPRRRIPGGVS–amide} \) by Nanotools, Teningen, Germany. Polyclonal antibodies were generated against the cytoplasmic domain of shrew-1 in rats in collaboration with Genovac, Freiburg, Germany. Secondary antibodies (FITC, Texas Red, Cyanine-5, Cy3) were from Jackson Immunochemicals (Dianova, Hamburg, Germany). Alexa fluor™ 466-labelled antibodies were purchased from Molecular Probes (Leiden, The Netherlands).

**Surface biotinylation experiment and immunoblots**

The cell surface of confluent monolayers was labelled on ice with 0.5 µg/ml membrane-impermeable EZ-Link Sulfo-NHS-Biotin (Perbio, Bonn, Germany) in PBS, pH 9.0. After quenching (50 mM ammonium chloride in PBS, 0.1 mM CaCl₂, 1 mM MgCl₂) the cells were lysed in 0.5 ml of RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.5, 0.25% sodium dodecyl sulphate, 0.1% Nonidet P-40) containing the protein inhibitor cocktail Complete (Roche, Germany) for 10 min at 4°C. Protein of each lysate was used for precipitation (16 h at 4°C) with 30 µl of Neutravidin beads (Perbio, Germany). The precipitates were washed as described previously (Rajasekaran *et al.*, 1999) and immunoblotted. Immunoblots were performed as described before (Kaufmann *et al.*, 2000).

**Cell culture, plasmid transfection and generation of stable cell line**

The human urinary bladder cell lines RT112 and EJ28 were described previously (Gaetje *et al.*, 1997). MCF-7 cells were obtained from ECACC (Salisbury, UK). EEC145T and 12Z were endometriotic cell lines established by our group as described previously (Zeitvogel *et al.*, 2001). Canine kidney epithelial cell line MDCK obtained from ATCC (ATCC CCL 34) was used to generate cell lines stably expressing shrew-1-GFP. All cell lines were cultured in Dulbecco’s Modified Eagle
medium containing antibiotics and 10% FCS. All cell culture reagents were purchased from Invitrogen (Karlsruhe, Germany). Transient transfections were performed in MCF7 using Polyfect transfection reagent from Qiagen (Hilden, Germany). MDCK cells were transfected using Effectene transfection reagent from Qiagen.

Immunofluorescence, confocal microscopy and antibody permeabilization assay

For immunofluorescence staining cells grown on glass cover slips were fixed in 4% paraformaldehyde and permeabilised by treatment with 0.2% Triton X-100 (both in PBS). Incubation with primary and secondary antibodies as well as visualization by immunofluorescence and CLSM was done as described before (Kaufmann et al., 2000). Image processing of 3D-data sets was performed with a Silicon Graphics (Mountain View, CA, USA) workstation (using ‘Imaris’ Bitplane AG, Zurich, Switzerland) 3D multi-channel image processing software (Messerli et al., 1993). Images were further processed using Adobe Photoshop.

For the antibody permeabilisation assay, MCF7 cells were transfected with shrew-1-GFP and grown to confluency. Cells were either fixed with 4% PFA or left unfixed (living samples). IF was performed as described.

Coimmunoprecipitation

Cells were washed twice with ice cold PBS and lysed for 30 min at 4°C in a buffer containing 10 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 60 mM n-octyl-glucoside. Samples were precleared for 1 h at 4°C using protein G-
sepharose (20 µl, 1:1) and subjected to immunoprecipitation overnight at 4°C using anti-GFP IgG (5 µg, mAb), anti-E-cadherin (5 µg, mAb 5H9), anti-Pan-cadherin (3 µg, followed by 2h incubation with protein G-Sepharose (30 µl, slurry 1:1). After 4-5 washes with the immunoprecipitation buffer, samples were separated by SDS-PAGE (12% acrylamide) and transferred to nitrocellulose. Immunoblotting was performed as described.

**In vitro “pull-down” binding assays**

β-catenin cloned in the expression vector pcDNA3.1 was synthesized by *in vitro* transcription-translation in the presence of 35S-methionine using the TNT™-coupled reticulocyte lysate (Promega, Mannheim). Glutathione S-transferase cytoplasmic domain of shrew-1 fusion (GST-CPD-shrew) (see plasmid constructs) was expressed in *E. coli* BL21 pLysS. Pull-down assays were performed as described previously (Kaufmann *et al.*, 2000).

**ACKNOWLEDGEMENTS**

We thank Reinhard Jahn and Joachim Kirsch for critical reading of the manuscript, Peer Bork and Gisbert Schneider for valuable support with the computational analysis, Karl-Friedrich Becker for pancreas tissue extracts, Ela Frye and Alex Schreiner for helpful discussions and their enthusiasm, Viktor Jakob, Beata Krebs and Heinz Schewe for expert technical assistance. Fellowships have been provided by the the Boehringer Ingelheim Foundation to S.Z. and the FAZIT Foundation to A.Z. and S.B. This work was supported by grants of the Deutsche Forschungsgemeinschaft (Sta 187/11-1-5; SFB 628), the Stifterverband der Deutschen Wissenschaft, and the Boehringer Ingelheim Stiftung.
REFERENCES


**Annu Rev Cell Biol, 4, 487-525.**


Kaufmann, U., Zupping, C., Waibler, Z., Rudiger, M., Urbich, C., Martin, B.,


FIGURE LEGENDS

Fig. 1. (A) Diagram depicting DDRT-PCR performed with invasive and non-invasive passages of the endometriotic cell line EEC145T, leading to the identification of shrew-1 mRNA. (B) The 391 bp cDNA was used as a probe to test for the presence of shrew-1 mRNA in endometriotic and carcinoma cell lines. Poly A⁺ RNA was prepared from the cell lines EJ28 (invasive bladder carcinoma), RT112 (non-invasive bladder carcinoma), EEC145T (p17 = invasive passage 17; p33 = non-invasive passage 33 of the endometriotic cell line) and Per 143T (peritoneal cells immortalised with SV40 T antigen). A Northern blot probed with ³²P-labelled shrew-1 probe detected an mRNA of about 4 kb in the invasive endometriotic cell line. Lower panel: the membrane was reprobed with cytochrome C oxidase to check the integrity and loading of the RNA samples. p17 and p33 are on the same blot at the same exposure-same as cyt c oxidase control.

Fig. 2. (A) The complete 411 amino acid sequence of the shrew-1 protein. The putative signal peptide is depicted in bold letters and the transmembrane domain is underlined. (B) lanes 1 and 2 show the endogenous expression of shrew-1 protein in pancreas and uterus sections, respectively, as detected by immunoblotting using the monoclonal antibody against shrew-1; lanes 3 and 4 show the autoradiography of in vitro translated luciferase control cDNA and shrew-1-BP, respectively after separation by SDS-PAGE; lane 5 depicts shrew-1-GFP expressed in MCF7 cells, as detected by monoclonal GFP antibody, lane 6 shows shrew-1-GFP detected by the rat polyclonal antibody against shrew-1 cytoplasmic peptide and lane 7 shows shrew-1-GFP detected by the monoclonal antibody generated against shrew-1 peptide of the putative ectodomain. (C) Immunoprecipitation of endogenous shrew-1 from uterus
cell extracts using shrew-1 polyclonal antibody and detection with the monoclonal antibody. Lane 2 depicts the input cell extract and lane 3 shows the immunoprecipitated protein detected with the monoclonal antibody. **(D)** Immunoprecipitation of endogenous shrew-1 from uterus cell extracts using shrew-1 monoclonal antibody and detection with the polyclonal antibody. Lane 2 depicts the input cell extract and lane 3 depicts the immunoprecipitated protein detected with the polyclonal antibody. Input is 10% of the total cell extract. Lane 1 in each C and D depicts the marker proteins.

**Fig. 3.** Membrane localization of shrew-1. Shrew-1 tagged with GFP (shrew-1-GFP) or BP (shrew-1-BP) was expressed in the eukaryotic epithelial cells: 12Z (human invasive endometriotic cell line), RT112 (human bladder carcinoma cell line, non-invasive), EJ28 (human bladder carcinoma cell line, invasive) and MCF7 (human breast carcinoma cell line, non-invasive). A-D show shrew-1-GFP fluorescence and E-H show immunofluorescence signals using a mouse monoclonal antibody against the BP tag visualized by a mouse-specific fluorochrome-conjugated secondary antibody. The arrows indicate the expression of shrew-1 at the membrane. The arrows depict the areas of cell-cell contact where shrew-1 is concentrated. The cells shown are transfected cells which border a few non-transfected cells.

**Fig. 4.** Cell surface biotinylation of MCF7 cells transfected with shrew-1-GFP. The biotinylated cell surface proteins were pulled down with neutravidin-coupled beads. The proteins present in various cell extract fractions were analysed by Western blots. **(A)** Shrew-1-GFP was detected by anti-GFP antibody (lanes 1-5). **(B)** E-cadherin, a positive control membrane protein, was detected by a monoclonal antibody against E-cadherin (lanes 1-4) and **(C)** Pyruvate kinase, a negative control cytosolic protein,
was detected with a specific antibody (lanes 1-4). UCX: untransfected cell extract, CX: transfected cell extract, sup: supernatant after pull-down of the biotinylated fraction, BF: pulled down biotinylated fraction, C: control of neutravidin beads bound to non-biotinylated cell extract.

**Fig. 5.** Carboxyl-terminus of shrew-1 is cytoplasmic. Shrew-1-GFP transfected MCF7 cells were permeabilised (A and B) or not permeabilised (C and D), as described in Material and Methods and then subjected to immunofluorescence staining with anti-GFP antibody and Alexa 594-labelled secondary goat anti-mouse antibody (B and D: red fluorescence). Intrinsic GFP fluorescence is green (A and C). Shrew-1-GFP could be detected in permeabilised cells by immunostaining with anti-GFP antibody (B) but not if the cells were not permeabilised (D).

**Fig. 6.** Colocalization of shrew-1-GFP with endogenous E-cadherin (red) at the membrane in MDCK cells (A-C); and in MCF7 cells (D) along the xy-axis as seen in the confocal microscope. Colocalization at the junctions is seen along the xz-axis with the confocal microscope (E, F).

**Fig. 7.** Interaction between shrew-1 and E-cadherin shown by coimmunoprecipitation. (A) MCF7 cells transfected with shrew-1-GFP (lanes 1, 3) or with GFP (lanes 2, 4) were subjected to immunoprecipitation with anti-GFP. In order to test antigen content, 10% of the total cell extract (Input) was immunoblotted (IB) with anti-GFP (upper panel) and anti-E-cadherin plus anti-β-catenin (middle panel) antibodies. Coimmunoprecipitations (Co-IP) were performed with anti-GFP antibody and the immunoprecipitates subjected to immunoblotting with anti-E-cadherin then
anti-β-catenin antibodies (lanes 3, 4). (B) In the reverse experiment, the cell extracts from MCF7 cells transfected with GFP (lanes 1, 3) or shrew-1-GFP (lanes 2, 4) were subjected to immunoprecipitation (IP) with anti-E-cadherin antibody. Input panels depict 10% of the cell extracts immunoblotted with anti-GFP antibody (upper panel), or endogenous E-cadherin protein immunoblotted with anti-E-cadherin antibody (lower panel). Coimmunoprecipitations (Co-IP) were performed with E-cadherin antibody, and shrew-1 was detected by immunoblotting with anti-GFP antibody as seen in lane 4. CX denotes the total cell extract. (C) Coimmunoprecipitation of N-cadherin and shrew-1-GFP. A: EJ28 cells were transfected with GFP (lanes 1, 3) or shrew-1-GFP (lanes 2, 4). Input shows 10% of the total cell extracts (lanes 1, 2). Immunoprecipitation (Co-IP) was performed with GFP-antibody (lanes 3, 4). Immunoblotting was performed with antibodies against GFP, N-cadherin and β-catenin. No interaction of shrew-1 with N-cadherin and β-catenin was observed.

(D) Direct interaction of β-catenin with the cytoplasmic domain of shrew-1 (GST-CPD-shrew) in an in vitro pull-down assay. Full-length β-catenin was translated in vitro using 35S methionine. GST and GST-CPD-shrew were purified on glutathione sepharose beads, then incubated at RT for 1 h with radioactively labelled β-catenin. After washing the beads, samples prepared as described in Material and Methods were subjected to SDS-PAGE and autoradiography. Lane 1: radioactive β-catenin as input, lane 2: the marker, lane 3: GST alone with β-catenin and lane 4: GST-CPD-shrew with β-catenin.

Fig. 8. Effect of scatter factor (SF) on MDCK cells. MDCK cells transfected with shrew-1-GFP were seeded at very low density on cover slips and were grown till formation of small colonies. SF/HGF was added at a concentration of 20 ng/ml to
these cells. and the effect was monitored on cover slips from the same culture dish at 0h, 4h, 8h and 15h. After 8h, cell-cell contacts were disrupted but colocalization of shrew-1-GFP (green) and endogenous E-cadherin (red) could also be seen in cytoplasmic vesicles. After 15h shrew-1-GFP and endogenous E-cadherin colocalised again predominantly at the plasma membrane.
Identification of shrew-1 mRNA from invasive cells

Bharti et al; Figure 1
Bharti et al; Figure 2
Bharti et al; Figure 3
Bharti et al; Figure 4
Bharti et al; Figure 6
Bharti et al; Figure 7