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A novel basement membrane-induced gene identified in the human endometrial adenocarcinoma cell line HEC1B

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Abstract Cultured HEC1B human endometrial adenocarcinoma cells respond to reconstituted basement membrane (Matrigel) by morphological and functional differentiation in vitro. Our goal is to identify genes involved in this differentiation process. By means of rt-PCR, we were able to isolate the novel 2.4 kb Matrigel-induced transcript *icb-1* containing an open reading frame predicting a 31.7 kDa protein. The time-dependent induction of *icb-1* gene expression by basement membrane was confirmed by Northern blot experiments. In a data bank search, several EST homologues corresponding to the 3′ untranslated region could be found. In summary, *icb-1* as a new tool enables us to study molecular mechanisms of cell-matrix interactions contributing to carcinogenesis.

Key words: Basement membrane; Endometrial adenocarcinoma; Extracellular matrix; Cell-matrix interaction; HEC1B cell

1. Introduction

Endometrial adenocarcinoma is the most frequently diagnosed malignancy of the female genital tract in the western countries [1]. The molecular mechanisms underlying carcinogenesis in the endometrium are still largely unknown. Particularly, the role of the extracellular matrices (ECMs), like the basement membrane, in carcinogenesis is not well understood. Components of the ECM are necessary for cell adhesion which is in turn involved in the regulation of proliferation, migration, tissue repair, cell shape and differentiation [2,3]. The importance of altered cell-matrix interactions for tumor progression is obvious. Tumor cells loose their typical cytoarchitecture, break through the basement membrane and invade the underlying stroma [4].

In vitro studies of endometrial carcinogenesis have been hampered by further dedifferentiation of normal and malignant endometrial cells grown under conventional culture conditions [5,6]. The loss of differentiation in vitro is accompanied by the loss of expression of tissue specific genes, like the expression of estrogen and progesteron receptors in endometrial adenocarcinoma cell lines.

Culturing the endometrial adenocarcinoma cell lines HEC1B and HEC1B(L) on top of a reconstituted basement membrane (Matrigel) leads to a more pronounced differentiated phenotype [7,8] if compared to cells cultured on plastic. In initial attempts to identify biologically important genes that are involved in this in vitro differentiation process, short cDNA fragments representing differentially expressed mRNA species [9] and differentially expressed secretory proteins [7] could be identified. In continuation of this work, the goal of this study was to identify genes regulated by the contact of HEC1B cells to a basement membrane, that could serve as potent markers to study the molecular basis of basement membrane-regulated differentiation of tumor cells, and elucidate the signal transduction pathways involved in this process.

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Abbreviations: kDa, kilodalton(s); SFDM, serum-free defined medium; bp, base pair(s); EST, expressed sequence tag; ECM, extracellular matrix; FCS, fetal calf serum

2. Material and methods

2.1. Cells and tissues

Tumor cell lines were from ATCC (Rockville, MD, USA) except of the Ishikawa line, that was kindly provided by Prof. Nishida (University of Tsukuba, Japan). 95003 uterine fibroblasts were prepared from clinical specimen as described previously [10]. Normal colon and colon carcinoma tissue was kindly provided by Dr. R. Broll (Clinic for Surgery, Medical University Luebeck). HEC1B, AN3-CA and Ishikawa are human endometrial adenocarcinoma cell lines. PC-3 and LNCAP are human prostata carcinoma cell lines, MCF-7 is a human mammary and BG-1 a human ovarian tumor cell line. HL60 is a human acute myeloid leukemia line that can be differentiated in vitro into granulocytoid cells by DMSO treatment [11]. MONO-MAC-1 is a human acute monocyte leukemia cell line.

2.2. Cell culture

HEC1B, AN3-CA, Ishikawa, HL60, BG-1, PC-3 and MONO-MAC-1 were grown in a humidified 5% CO_2 atmosphere at 37°C in Dulbecco's modified Eagle's medium (DMEM) without phenol red (Serva, Heidelberg, Germany) supplemented with 10% (v/v) fetal calf serum (FCS) and antibiotics (penicillin 100 U/ml, streptomycin 100 mg/ml (Sigma, Deisenhofen, Germany)). LNCAP were cultured with 15% FCS, and to MCF-7 cells 1 mM sodiumpyruvate was added. After one passage the cells were harvested by trypsin-EDTA treatment, washed several times with PBS and resuspended in serum-free defined medium (SFDM), which consisted of DMEM/F12, 2 μg/ml insulin, 10^{-8} M hydrocortision, 2×10^{-8} M sodium selenite, 1 μg/ml putrescine and 40 μg/ml transferrin (all Gibco BRL, Eggenstein, Germany). 300 000 cells per well were cultured on top of a layer of 230 μl Matrigel (Serva, Heidelberg, Germany) in 24-well plates for 24, 48, 72 and 96 h. For comparison the cells were cultured on plastic in the presence of DMEM containing 10% FCS or in the presence of SFDM, respectively.

2.3. RNA isolation and rt-PCR

Total cellular RNA was isolated using the standard guanidinum isothiocyanate gradient centrifugation method [12]. Cells from approximately 80% confluent cultures were harvested by trypsin-EDTA treatment and resuspended in homogenization buffer. Cells grown on Matrigel were directly lysed. Tissues were destroyed in liquid nitrogen using a dismembrator and the tissue powder was processed as described for cells. To isolate cDNA from its 3′-end, total cellular RNA (1 μg) was reverse transcribed using AMV reverse transcriptase (Gibco BRL, Eggenstein, Germany) and 1.8 μM oligo dT anchor primer (5′-GACCACGCGTATCGATGTCGAC dT_{16}-3′). PCR reactions were performed in 1×PCR buffer, 1.5 mM $MgCl_2$,

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2.5 μM of each dNTP, 0.25 μM hexamer random, or PCR anchor primer (5′-GACCACGCGTATCGATGTCGAC-3′) (Boehringer, Mannheim, Germany), 1 U Taq-Polymerase and 10% (v/v) cDNA. 5′ RACE was performed using the 3′/5′ RACE Kit (Boehringer, Mannheim) and the *icb-1* specific primers 5′-CCTCTCGCCCTCA-CAGTCCTTTG-3′ and 5′-CAGCAGGAGGGTTTGATAA-3′. The cDNA was amplified in 35 cycles (1 cycle = 1 min 94°C melting, 2 min 55 to 62°C annealing, 3 min 72°C extension). To avoid false results by DNA contamination, the PCR was performed with RNA as negative controls. Using agarose gel electrophoresis, rt-PCR products obtained from RNA of cells grown on Matrigel were compared to those products generated from RNA isolated of cells grown on plastic. Differentially expressed fragments were cut out, tested by reamplification using nested PCR, directly cloned using the TA-cloning kit (Invitrogen, The Netherlands), and finally sequenced. In the case of the sequence reported here, the corresponding cDNA was subcloned into the pUC18 vector and the identity was confirmed by sequencing both strands.

2.4. Northern blot analysis

Ten μg RNA, obtained from each cell culture condition, was separated on a 0.9% agarose gel containing 2.2 M formaldehyde. RNA was then vacuum blotted onto Hybond$^+$ nylon membranes (Amersham, Braunschweig, Germany) and cross-linked by UV irradiation. The DNA fragments used as probes were released from the inserted pCRII vector by restriction digestion, purified by Gene clean and labeled with ^{32}P-dCTP using a random primer labeling kit (both Dianova, Hamburg, Germany)

3. Results and discussion

3.1. Isolation and sequence analysis of a human basement membrane-induced cDNA clone

The 3′ cDNA fragments obtained by rt-PCR of total RNA isolated from HEC1B cells cultured either in SFDM on plastic or on Matrigel were compared by agarose gel electrophoresis. A fragment recognizing an mRNA with a matrix-induced expression pattern (Fig. 1) was amplified by specific binding of the PCR anchor primer to the 3′-end and its random annealing at the 5′-end of the cDNA instead of binding of a hexamer random primer. Isolation and sequencing of clone log-ot4 revealed a cDNA with the length of 2366 bp (Fig. 2). Since 5′ RACE experiments did not succeed in enlargement of the isolated cDNA clone and its size is consistent with the detected mRNA, we propose the completeness of the sequence reported here. It contained a significant open reading frame with seven putative translation initiation codons and a stop codon at position 981. The start codon at position 129 is most consistent with Kozak's rule [13]. The gene represented by the open reading frame was named *icb-1* (induced by contact to basement membrane). A sequence analysis revealed a significant polyadenylation signal in the expected distance from the 3′-end [14]. The absence of repeated ATTTA motifs suggests a relative high mRNA stability [15]. In a DNA data bank search, no homologues to the coding region were found, but 24 expressed sequence tags (ESTs) from various cell lines and tissues showed a high homology ($>95\%$) to the 3′ untranslated region of cDNA clone log-ot4. Most of these ESTs are expressed in tumors or fetal tissues, suggesting an association of *icb-1* with cell proliferation. The open reading frame predicts a 31.7 kDa protein, to which in a protein data bank search no molecules with a homology $>50\%$ could be found. In a protein sequence analysis, two 4-residue patterns for nuclear targeting were found. Since no significant transmembrane helices, mitochondrial targeting sequences or motifs for peroxisomal proteins or other motifs for intracellular sorting could be identified, most likely the predicted protein is localized in the nucleus.

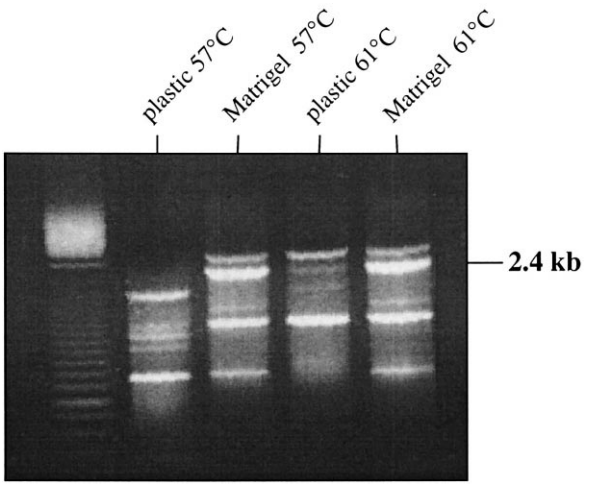

Fig. 1. Display of differentially expressed mRNA molecules amplified with rt-PCR. Compared are products obtained from RNA of cells cultured on plastic or Matrigel (both in SFDM), which were analyzed at two different annealing temperatures.

3.2. Matrix regulation of icb-1

To confirm the basement membrane-dependent differential expression of *icb-1* shown by rt-PCR, total RNA was extracted from HEC1B cells cultured on Matrigel in SFDM for different time periods (24, 48, 72 and 96 h). For this Northern blot experiment (Fig. 3), different probes were used: a 349 bp fragment representing the 5′-end of log-ot4 from position 18 to 367, and a 695 bp probe representing the 3′-end of the cDNA from position 1429 to 2123. The 5′ probe detected a significant increase of the *icb-1* steady-state mRNA level after 24 h culture on basement membrane up to 4.4-fold with a time-dependent decrease to a 1.6-fold induction after 96 h. Probing with the 3′ fragment shows the same pattern: a significant induction peak after culture periods of 24 and 48 h on Matrigel and a decrease thereafter. Neither the 3′ nor the 5′ probe visualized differences in the steady-state mRNA *icb-1* levels if cells were cultured on plastic irrespective of the medium used (10% FSC or SFDM). In comparison with the size of the 18S and 28S rRNA, the size of the detected mRNA was judged to be approx. 2.5 kb, which is consistent with the cDNA length. In summary, steady-state *icb-1* mRNA levels are significantly induced by culturing HEC1B cells on a reconstituted basement membrane. If compared to data published for either normal breast cells [16] or endothelial cells [17], it is tempting to speculate that integrins are responsible for the transformation of the information provided by basement membrane into an intracellular signal and ultimatively resulting in alteration of gene expression. Since many signal transduction pathways have been described to be triggered by integrins [18,19], and particularly since even matrix responsive elements have been described within the β-casein promoter [20] and the tenascin-c promoter [21], we propose that cloning of *icb-1* promoter sequences will enable us to elucidate signalling cascades triggered by components of the basement membrane presumably via integrin mediated pathways.

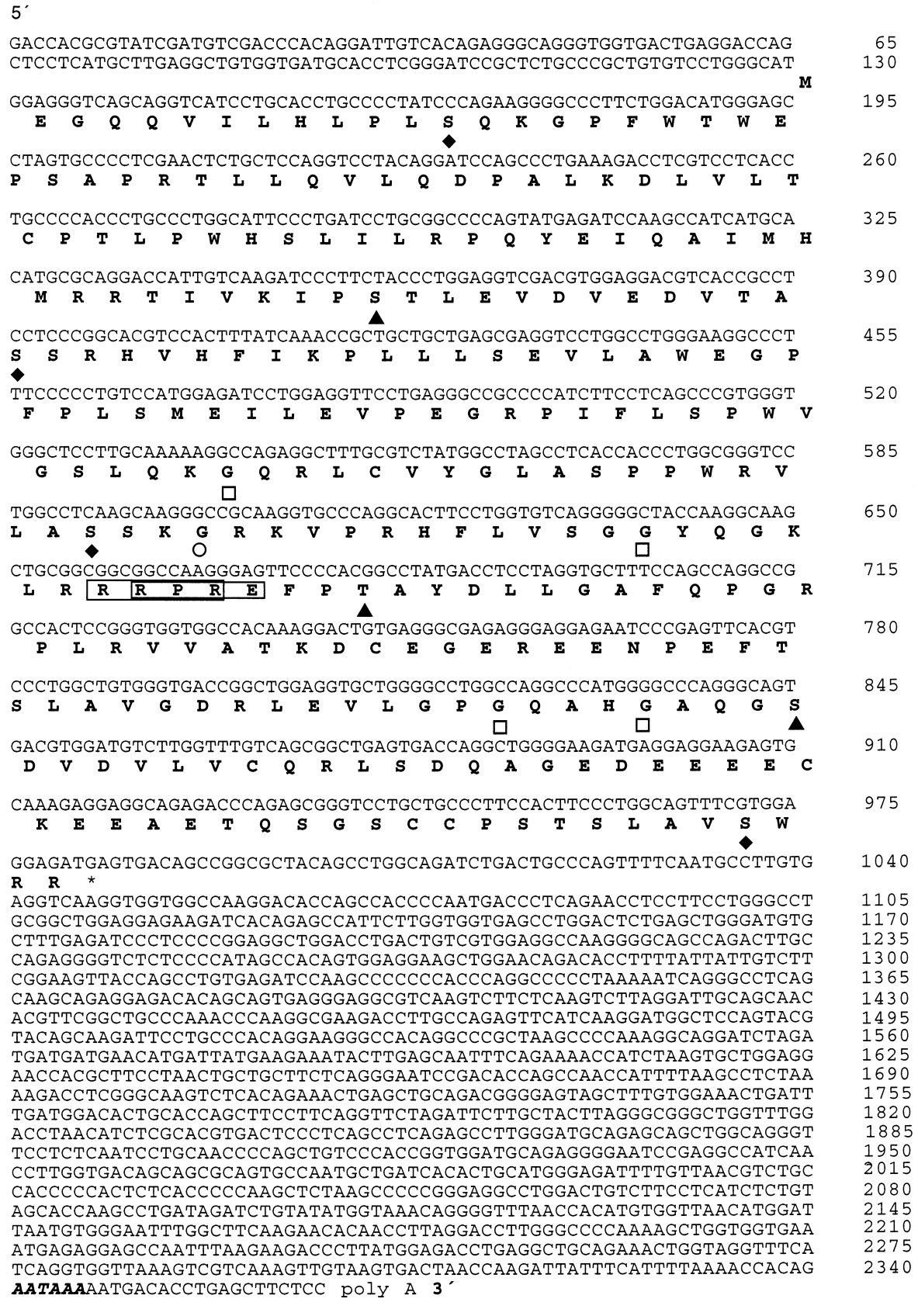

Fig. 2. Nucleotide and deduced amino acid sequences of human *icb-1* cDNA, clone log-ot4. ♦: Protein kinase C phosphorylation site. ▲: Casein kinase II phosphorylation site. □: N-myristoylation site. ○: Amidation site. Boxed: Two 4-residue patterns for nuclear targeting. Bold: Polyadenylation signal. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AF044896.

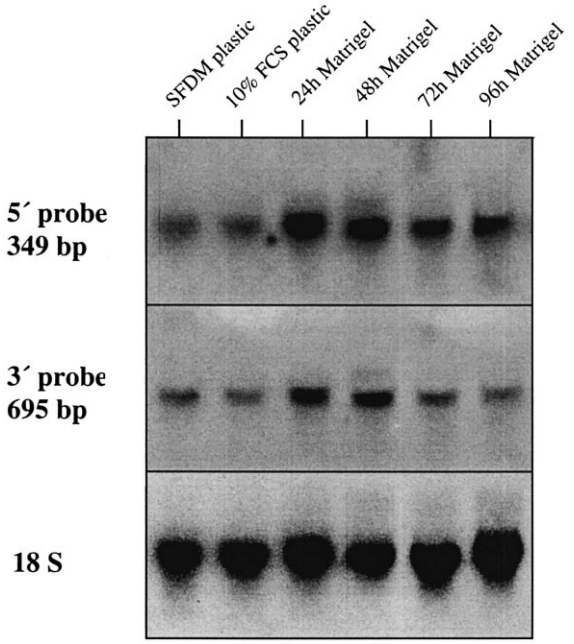

Fig. 3. On a Northern blot, probes representing the 3′- as well as the 5′-end both detect a significant induction of *icb-1* mRNA following 24 h culture of HEC1B cells on a reconstituted basement membrane (Matrigel+MG).

3.3. Tissue specificity of icb-1 expression

Using rt-PCR and Northern blot analysis, the expression of *icb-1* mRNA was studied in nine different human cell lines (cultured in SFDM on plastic) and two human tissues. RNA isolated from these different sources was reversed transcribed using hexamer random primers and amplified with *icb-1* specific primers (25-mers). The results of rt-PCR, showing a 475 bp *icb-1* specific fragment in the case of positive expression (Fig. 4), could be verified by Northern blotting (data not shown). To verify absent *icb-1* expression in two cell lines and to test cDNA quality, a 320 bp γ-actin specific fragment was amplified as a positive control. The results show *icb-1* mRNA expression in all tested tissues and cell lines except in the prostata cancer cell line LNCAP and the ovarian cancer cell line BG-1. Interestingly, both cell lines share a common feature, they both originate from moderately differentiated tumors and they both express steroid hormone receptors in culture. Since the steroid receptor positive cell line MCF-7 expresses *icb-1*, the question whether or not expression of steroid hormone receptors is involved in downregulation of *icb-1* by contact to basement membrane is subject of further investigations.

3.4. Conclusion

We have succeeded in isolating the novel human gene *icb-1* predicting a 31.7 kDa protein. Increased transcription of *icb-1* is induced by contact of HEC1B endometrial adenocarcinoma cells to a reconstituted basement membrane. *Icb-1* is expressed in different endometrial adenocarcinoma cell lines and various other human cell lines derived from solid tumors or from leukemia cells. *Icb-1* or its promoter elements could represent a molecular endpoint of either an outside-in signal transduction pathway triggered by cell contact to the basement membrane or by ECM-induced alteration of cellular phenotype. *Icb-1* represents a potential downstream marker which will enable us to elucidate specific basement membrane-dependent signalling cascades that may play an important role in carcinogenesis.

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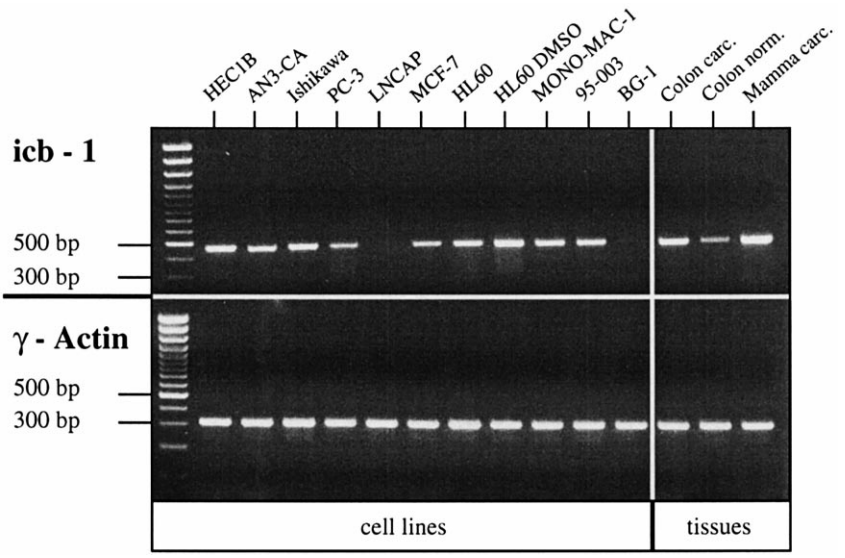

Fig. 4. *Icb-1* specific rt-PCR of RNA isolated out of different cell lines and tissues resulted in amplification of a 470 bp fragment. As primers, the 23-mers L1981 5′-ATCTATCAGGCTTGGTGCTACA-3′ and U1530 5′-AACCACGCTTCCTAACTGCTGCT-3′ were used. A specific 320 bp γ-actin fragment was amplified as a positive control using the oligonucleotides actF 5′-ATCATGTTTGAGACCTTCAA-3′ and actR 5′-CATCTCTTGCTCGAAGTCCA-3′ as primers.

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