HERC5, a HECT E3 ubiquitin ligase tightly regulated in LPS activated endothelial cells

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Summary

By differential screening we isolated genes upregulated in inflammatory cytokine-stimulated human skin microvascular endothelial cells. One of these cDNAs encoded RCC1 (regulator of chromosome condensation 1)-like repeats and a HECT (homologous to E6-AP C-terminus) domain, representing a member of the HERC (HECT and RCC1 domain protein) family of ubiquitin ligases. The mRNA level of this member, *HERC5*, is specifically upregulated in endothelial cells by the pro-inflammatory cytokines tumor necrosis factor α and interleukin 1β, and by lipopolysaccharide (LPS), but is hardly expressed in other cells of the vascular wall such as primary smooth muscle cells and fibroblasts. Regulation of *HERC5* gene expression suggests a critical role for the transcription factor NF-κB. In contrast to mRNA expression HERC5 protein is subject of enhanced degradation upon LPS stimulation of endothelial cells. The time course of LPS-induced changes in HERC5 protein and mRNA levels suggests that the initial drop in HERC5 protein is balanced by increased protein synthesis due to upregulation of *HERC5* mRNA. This leads to recovery of HERC5 protein levels within 12 hours of LPS stimulation and points at a tight control of HERC5 protein. To analyze functional activity of this putative member of the ubiquitin-conjugating pathway we performed in vitro assays with different ubiquitin-conjugating enzymes. We found that HERC5 possesses ubiquitin ligase activity and requires the presence of the ubiquitin-conjugating enzyme UbcH5a for its activity. These data show for the first time that a functionally active HECT ubiquitin ligase exhibits a tightly controlled cytosolic level under inflammatory conditions in endothelial cells.

Key words: Endothelial cells, LPS, Inflammation, NF-κB, Ubiquitin ligase

Introduction

A healthy organism reacts with help of the immune system and a process termed inflammation against microbial infection in order to restore the integrity of the affected tissue. Inflammation is either resolved or can progress towards a chronic state favoring pathologies such as atherosclerosis and rheumatoid arthritis, or even malignancies (Coussens and Werb, 2002; Nathan, 2002). Bacterial endotoxin itself and/or cytokines released from immune cells recruited to the site of injury trigger activation of endothelial cells (ECs) (Bierhaus et al., 2000). Subsequently, altered gene expression of chemokines, cytokines, proinflammatory enzymes, cell surface receptors, adhesion molecules, and components of the complement and coagulation cascades leads to recruitment and activation of immune cells (Bierhaus et al., 2000).

ECs are activated by pro-inflammatory cytokines, including tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β), and by bacterial lipopolysaccharide (LPS) through Toll-like receptors (Akira et al., 2001; Suzuki et al., 2001; Zhang and Ghosh, 2000) signaling predominantly through the NF-κB transduction pathway (Li and Verma, 2002; Yamamoto and Gaynor, 2001). Activation of this pathway is achieved by phosphorylation of inhibitor of NF-κB (IκB) by the IκB kinase (IKK) complex (Ghosh and Karin, 2002; Ghosh et al., 1998). Phosphorylated IκB is ubiquitinated by the $SCF^{\beta TrCP}$ complex to target it for degradation by the 26*S* proteasome (Brown et al., 1995; Chen et al., 1995). Subsequently, the released transcription factor NF-κB translocates into the nucleus, binds to κB consensus sites and induces gene expression together with other transcription factors also activated by inflammatory stimuli (De Martin et al., 2000).

In addition to transcriptional regulation, protein degradation seems to play a crucial role in the induction and modulation of the inflammatory response. The ubiquitin proteasome system is the major pathway for specific intracellular protein degradation and plays a crucial role in many cellular processes (Weissman, 2001). Proteins modified by polyubiquitination are targeted for proteasomal degradation (Thrower et al., 2000). Monoubiquitination serves other functions such as membrane transport or transcriptional regulation (Hicke, 2001). Ubiquitination of proteins involves a hierarchical enzymatic cascade consisting of a ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2) and, in most cases, ubiquitin ligases (E3) (Hershko et al., 2000; Pickart, 2001;

Weissman, 2001). Although only one E1 enzyme is functionally active and the number of E2 enzymes is limited, the number of E3 enzymes is abundant, thereby allowing for substrate specificity. Ubiquitin ligases can be identified by the presence of either a HECT (homologous to E6-associated protein C-terminus) domain (Huibregtse et al., 1995), a RING (really interesting new gene) domain (Lorick et al., 1999) or a U-box (Aravind and Koonin, 2000). So far, only RING ubiquitin ligases involved in inflammation have been identified (Deng et al., 2000; Ohta et al., 1999; Suzuki et al., 2001; Yang et al., 2000).

By differential screening of cytokine-stimulated ECs we identified HERC5, a ubiquitin ligase of the HERC family. We demonstrate its enzymatic activity and specific transfer of ubiquitin via UbcH5a. We further show that *HERC5* gene expression is upregulated in activated ECs in an NF-κB-dependent manner. HERC5 protein, however, is subject to enhanced degradation upon stimulation of ECs.

Materials and Methods

Cloning of *HERC5*

Differential screening of ECs was performed as described (Stehlik et al., 1998a). A partial cDNA clone, #881, encoding a HECT domain was elongated by cDNA library screening. For this purpose, a library was made from pooled mRNA isolated from human skin microvascular ECs (HSMECs) stimulated with either TNFα or LPS for various time points according to a cDNA library construction protocol (Stratagene). For cloning of the 5′-end a G-tailing strategy (Gibco) and SMART technology (Clontech) were applied. All PCR fragments were cloned into pGEM-T easy vectors (Promega), sequenced and analyzed using Sequence Navigator and MacVector software. The cDNA sequence of 3529 bp with an ORF of 1024 aa, initially termed *Croco*, was renamed *HERC5*.

For cloning of the promoter, BAC clones containing the *HERC5* genomic region were obtained. *Bam*HI/*Bgl*II restriction fragments of these BAC clones were subcloned into pBluescript and transformants were screened for *HERC5* exons. Two clones were isolated containing the 5′UTR sequence of *HERC5* and additional upstream sequence (GenBank AY532389). The genomic region upstream of the cDNA start site was analyzed for potential promoter activity and transcription factor binding sites using the following programs: Promoter Scan, Dragon Promoter Finder, TESS (Transcription Element Search Software) and Transfac database.

The *HERC5* cDNA start (clone GenBank AY337518) was defined as +1 and a fragment corresponding to –1569 to +36 was cloned into the luciferase expression vector pUBT-luc and reporter assays were performed as decribed previously (Hofer-Warbinek et al., 2000) using the Dual-Luciferase Reporter Assay system (Promega).

Cell culture

Primary HSMECs, human umbilical vein ECs (HUVECs), human smooth muscle cells (HSMCs) and skin fibroblasts (FBs) were isolated as described (Wojta et al., 1993; Wojta et al., 1992; Zhang et al., 1996). All cells were grown on gelatin-coated cell culture flasks at 37°C and 5% CO_2. HSMCs and FBs were cultured in M199 supplemented with 10% bovine calf serum (Hyclone), 2.2 mM L-glutamine, 80 units/ml penicillin, 80 μg/ml streptomycin and 0.2 μg/ml fungizone (all BioWhittaker). ECs were cultured in M199, 20% serum, 50 μg/ml endothelial growth factor supplement (Technoclone, Austria), antibiotics and 3 units/ml heparin (Roche). Primary cells were split in a 1:3 ratio and used up to the sixth passage. An EC line, HM2, (R. Hofer-Warbinek, personal communication) was generated by infection with an amphotrophic helper-free recombinant retrovirus construct, pLXSN16 E6/E7, containing the E6/E7 open reading frame of human papilloma virus 16 (Fontijn et al., 1995) and cultured under selection of 100 μg/ml G418 (Sigma). Confluent monolayers were left for 48 hours and subsequent experiments were done without changing the cell culture medium. Quiescence and response to activation of ECs was confirmed by determining either IL-8 secretion or E-selectin (ELAM-1) expression. The following agents were added per ml of medium: TNFα 500 units, LPS 600 ng, IL-1β 300 units, IL-6 200 units, IL-8 10 ng, IL-10 2 ng, EGF 10 ng, VEGF 10 ng, TGFβ 2 ng, CHX 10 μg. LPS and CHX were obtained from Sigma, all other agents were from Genzyme. Adenoviral infection of ECs with either recombinant adenovirus IκBα or control virus was performed as described previously (de Martin et al., 1997; Wrighton et al., 1996).

RNA isolation, northern blot analysis and RT-PCR

For RNA isolation, cells were washed, lysed directly in TRIzol reagent (Life Technologies) and total RNA was precipitated according to the manufacturer's protocol. For Northern blotting, 10 μg of total RNA each were separated on agarose/formaldehyde gels, transferred to GeneScreen *Plus* membranes (Du Pont) and hybridized with 10^6 cpm/ml of [α-^{32}P]dATP-labeled cDNA probes (Brostjan et al., 1997). The following HERC5 probes were used: bp 2890-3530 and bp 1-411. Blots were reprobed with both IκBα and IL-8 probes. Equal loading of RNA was confirmed by rehybridization with a GAPDH probe. Signals were analyzed with a PhosphorImager using ImageQuant software (Molecular Dynamics). The multiple tissue blot was hybridized according to the manufacturer's protocol (Clontech). Quantitative real-time RT-PCR was performed essentially as described previously (Kadl et al., 2002). Extracted RNA (High Pure RNA Isolation, Roche) was reverse transcribed using Superscript II (Invitrogen) and hexamer primers. Tissue RNA was obtained from Clontech. β_2-microglobulin (β2M) primer pair: 5′-gatgagtatgcctg-ccgtgtg-3′ and 5′-caatccaaatgcggcatct-3′; HERC5 primer pair: 5′-gggatgaaagtgctgaggag-3′ and 5′-cattttctgaagcgtccaca-3′. Primers were obtained from Invitrogen and RT-PCR efficiency was 1.82 for the β2M primer pair, and 2.17 for the HERC5 primer pair, respectively.

Antibody production and testing

The plasmid pQE32-HERC5-CT encoding the C-terminus of HERC5 (aa 659-1024) was used to express 6×His-tagged HERC5-CT protein in *E. coli* M15 and the recombinant protein was purified according to standard protocols (Qiagen). Immunization of rabbits was carried out according to standard immunization protocols. For testing of immune sera in immunoprecipitation, proteins were synthesized in a TNT-coupled transcription/translation system (Promega) in the presence of L-[^{35}S]methionine. 50 μl aliquots were diluted with lysis buffer (1% Nonidet P-40, 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$) and pre-cleared with protein A-Sepharose (Pharmacia). Samples were split and precipitated with 2 μl of either pre-immune or immune serum. After washing, samples were separated by SDS/PAGE. Fixed and dried gels were exposed and analyzed by autoradiography. Positive reacting serum was termed anti-HERC5 antiserum and used for further experiments.

Cell labeling, immunoprecipitation and pulse-chase

Quiescent ECs (5×10^6 cells per sample) were metabolically labeled with 20 μCi/ml L-[^{35}S]methionine or Promix (Amersham) in deficient RPMI 1640 medium (Gibco) supplemented as decribed above, but serum was dialysed against PBS. The presence of LPS in the labeling medium is indicated. For protein half-life measurements, metabolically labeled cells were washed and incubated in the presence of a 100-fold excess of unlabeled methionine/cysteine (chase) for the indicated times in the absence or presence of LPS. Protein extracts

were prepared on ice in 1% Nonidet P-40, 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 20 μg/ml phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Roche). Samples normalized to equal amounts of trichloroaceticacid-precipitable radioactivity were precipitated with 20 μl of anti-HERC5 antiserum and protein A-Sepharose (Pharmacia) according to standard protocols. After washing three times with buffer B (10 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.2% Nonidet P-40 and 2 mM EDTA), twice with buffer C (buffer B but 500 mM NaCl) and once with 10 mM Tris/HCl pH 7.5, samples were separated by SDS-PAGE and gels were analyzed using a PhorphorImager and ImageQuant software for quantification (both Molecular Dynamics).

E2 preparation and testing

Genes encoding the E2 enzymes were subcloned into bacterial expression vectors and fused to either a His-tag (H3, H5b, H5c, H8) or a GST-tag (H5a, H6, H7). pT7-7-H10 was expressed as described (Townsley et al., 1997). Expression conditions were optimized and bacterial lysates were prepared. Therefore, 50 ml of bacterial culture overexpressing a particular E2 enzyme were lysed in 1 ml of lysis buffer (50 mM Tris/HCl pH 7.5, 1% Triton X-100, 20 μg/ml PMSF) and sonicated. Supernatants were tested for thioester bond formation essentially as described (Jensen et al., 1995), but using GST-ubiquitin in the assay and subsequent detection by anti-GST antibody (Zymed) and a HRP-labeled secondary antibody (Amersham). Active enzyme preparations were used for further experiments.

Thioester bond formation assay

A fragment encoding aa 613-1024 of HERC5 was cloned into a pcDNAFlag vector (constructed by cloning an oligo encoding a Flag epitope into the *Hin*dIII-*Bam*HI site of pcDNAHisA) and the C994A mutation was introduced by PCR (Ho et al., 1989). The thioester assay was performed as described (Huibregtse et al., 1995). Where indicated, 2-4 μl of bacterially expressed E2 enzymes were added and samples were incubated for 20 minutes. GST-ubiquitin was expressed in *E. coli* DH5α and purified as described (Scheffner et al., 1993).

Statistics

Results, when applicable, are given as means±s.d. and significance was assigned to *P*-values of <0.05 (paired or unpaired *t*-test and one-way analysis of variance followed by Dunnet's test as post-test, respectively). Regression analysis was performed using non-linear regression curve fit. All statistic analyses were done using the GraphPad Prism3 Program.

Results

Identification and cloning of HERC5

Differential screening of a cDNA library to identify genes expressed in LPS- and TNFα-activated ECs, but not in quiescent ECs, resulted in several cDNA clones. Among those, a sequence encoding a HECT domain protein was identified owing to its homology to the C-terminus of E6-associated protein (E6-AP). The originally identified clone was elongated to 3529 bp by cDNA library screening and specific 5′-RACE PCR methods. An open reading frame encoding 1024 amino acids (aa) was determined containing a RCC1-like domain (RLD) at the N-terminus, separated from the HECT domain by a region of 275 aa without homology to any known protein domain (Fig. 1A). Members of the HECT family containing an RLD are designated HERC (HECT and RCC1 domain proteins). The clone identified by us, initially termed Croco

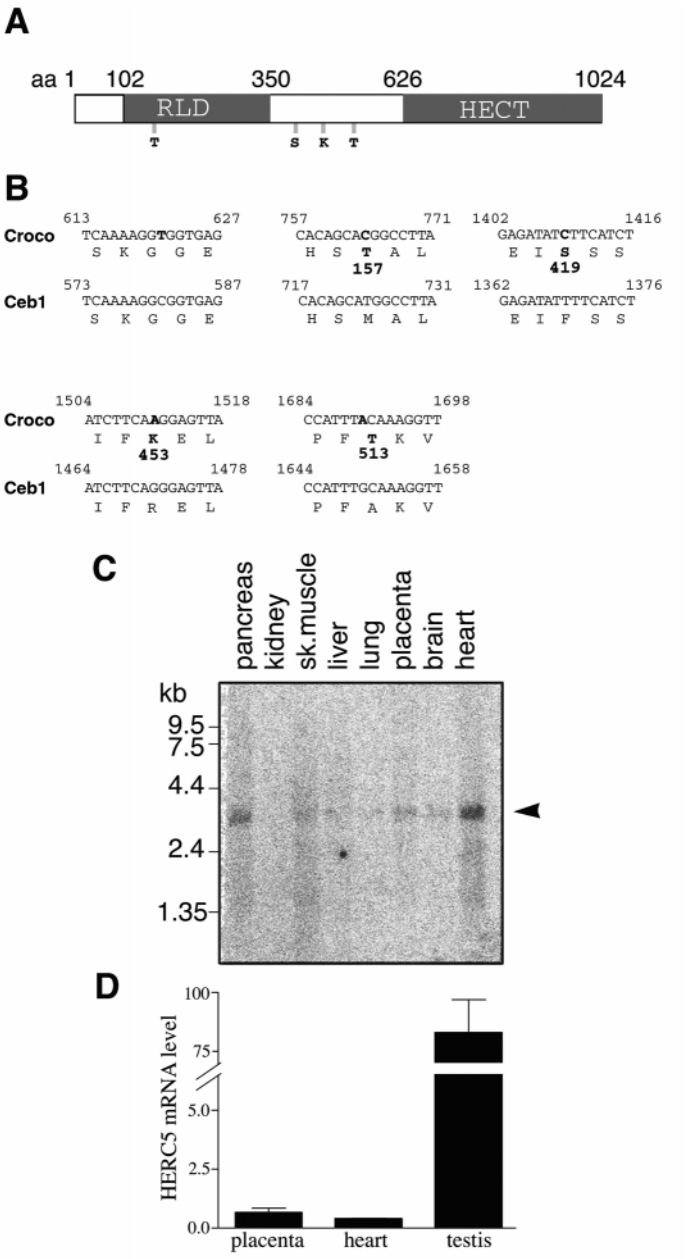

Fig. 1. Structure and expression of HERC5. (A) Schematic domain structure of HERC5. Amino acids in Croco different from those in Ceb1 are shown as grey bars. aa, amino acid; HECT, homologous to E6-AP C-terminus; RLD, RCC1-like domain. (B) Comparison of *Croco* and *Ceb1*. Differences in the cDNA and protein sequences are indicated in bold. Amino acids are given in the single-letter code and positions of amino acids are indicated below. GenBank accession numbers: Ceb1, AB027289; Croco, AY337518. (C) *HERC5* mRNA expression in human tissues. A commercially available multiple tissue blot containing poly(A)$^+$ RNA derived from different human tissues was probed for *HERC5* transcripts. sk. muscle, skeletal muscle. (D) Quantification of *HERC5* mRNA in selected tissues. Quantitative real-time RT-PCR was performed to quantify *HERC5* expression in heart, placenta and testis normalized to β_2-microglobulin expression.

(GenBank accession number AY337518), is very similar to the cyclin E binding protein 1 (*Ceb1*) (Mitsui et al., 1999) and was

classified as *HERC5* according to human genome approved nomenclature. The two sequences differ by a one base pair deletion in the 5′-UTR, by an additional 40 bp at the 5′-UTR of *Croco*, and five point mutations resulting in exchange of four amino acids. As shown in Fig. 1B, aa M157, F419, R453 and A513 of Ceb1 are changed to T, S, K and T, respectively, in Croco.

Tissue- and cell-specific expression of *HERC5*

By hybridization of a multiple tissue blot with a HERC5-specific probe we detected *HERC5* mRNA transcripts of approximately 3.7 kb in heart and pancreas. Weak signals are detectable in placenta and skeletal muscle (Fig. 1C). High *Ceb1* expression was reported for testis tissue (Mitsui et al., 1999), therefore we compared *HERC5* mRNA levels in placenta and heart to testis by quantitative real-time RT-PCR. *HERC5* mRNA level in testis was about 100 times higher than in placenta and heart tissue when normalized to β_2-microglobulin expression (Fig. 1D). We were interested in *HERC5* expression in ECs and detected *HERC5* transcripts in LPS-activated primary human skin microvascular ECs (HSMECs), human uterus microvascular ECs (HUMECs) and the HSMEC-derived cell line HM2. This cell line reflects endothelial cells in terms of inducible expression of adhesion molecules, LDL-uptake, and tube formation (R. Hofer-Warbinek, personal communication). As shown in Fig. 2A,B, *HERC5* mRNA is hardly detectable in quiescent cells by northern blot analysis, whereas upon LPS stimulation signals are detectable between 6 and 16 hours. Activation of microvascular ECs by TNFα revealed both a similar time course and a similar extent of *HERC5* mRNA expression (data not shown). Apart from microvascular ECs we also analyzed ECs derived from large vessels. By northern blot analysis we detected *HERC5* mRNA expression in primary human aortic ECs (HAECs) upon treatment with LPS for 6 hours, but not in primary human umbilical vein ECs (HUVECs) upon LPS or TNFα stimulation up to 24 hours (Fig. 2B and data not shown). No signals were detected in other cell types present in the vascular wall, such as skin fibroblasts (FBs) or human smooth muscle cells (HSMCs) after TNFα stimulation for up to 24 hours (Fig. 2B and data not shown). Additionally, *HERC5* expression in vascular cells was analyzed by quantitative real-time RT-PCR and normalized to β_2-microglobulin expression. The upregulation of *HERC5* mRNA level is approximately tenfold in HSMECs (Fig. 2C) and, owing to better sensitivity of this method, inducibility of *HERC5* mRNA in both HUVECs and HSMCs upon LPS stimulation was detectable (for 9 hours $P<0.01$, $n=4$). As shown in Fig. 2D, both basal and induced levels of *HERC5* mRNA in HUVECs and HSMCs are significantly lower than in HSMECs. In contrast to primary cells, *HERC5* mRNA levels were not inducible upon TNFα treatment in selected cell lines of either epithelial, haematopoietic, lymphatic or melanocytic origins (data not shown).

Regulation of *HERC5* mRNA expression

Having identified *HERC5* as an LPS-inducible gene in ECs, we tested whether cytokines targeting ECs would induce *HERC5* mRNA expression. Only the inflammatory stimuli LPS, TNFα, and IL-1β caused pronounced upregulation of *HERC5* mRNA levels in microvascular ECs after 8 hours of treatment (Fig. 3A). To analyze whether de novo protein synthesis is required for *HERC5* mRNA regulation, we used cycloheximide (CHX). As shown in Fig. 3A, no upregulation of *HERC5* mRNA was seen in the presence of CHX. These data indicate that *HERC5* mRNA upregulation by inflammatory stimuli is dependent on de novo protein synthesis in ECs. The transcription factor NF-κB is a central mediator of gene regulation in inflammation and is activated by TNFα, IL-1β and LPS (De Martin et al., 2000). Adenovirus-driven overexpression of IκBα, the inhibitor of NF-κB, abolishes NF-κB-dependent upregulation of inflammatory genes such as IL-1, IL-6, IL-8 and VCAM-1 in

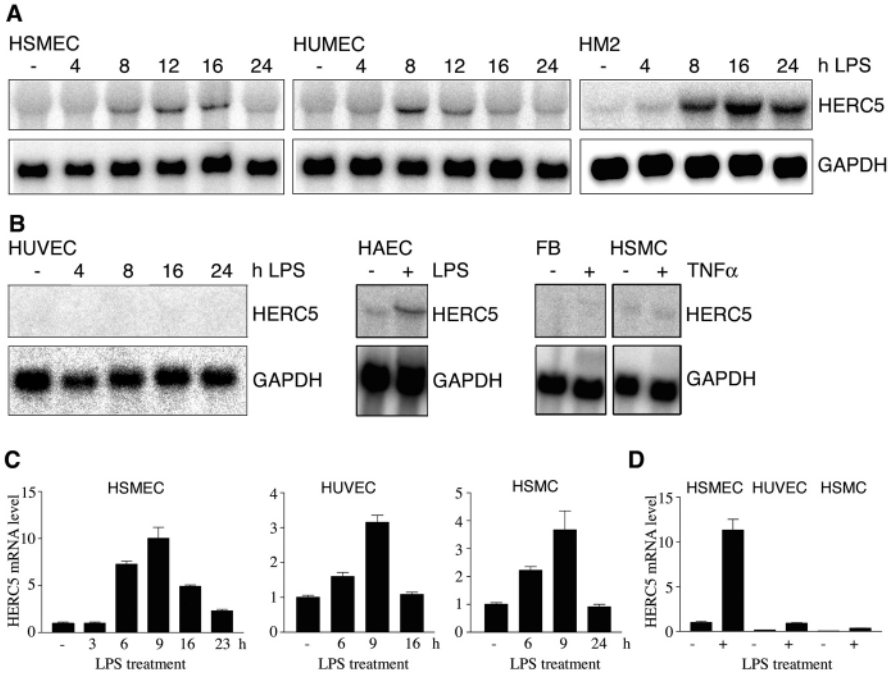

Fig. 2. *HERC5* mRNA expression in vascular cells. (A,B) Total cellular RNA from human ECs of different origin stimulated with either LPS or TNFα for the indicated periods of time was probed for *HERC5* mRNA by northern blotting. HAEC were stimulated for 6 hours, FB and HSMC were stimulated for 9 hours. FB, human skin fibroblasts; HAEC, human aortic endothelial cells; HM2, a microvascular endothelial cell line; HSMC, human smooth muscle cells; HSMEC, human skin microvascular endothelial cells; HUMEC, human uterus microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells. (C) *HERC5* expression analysis by quantitative real-time RT-PCR. Cells were stimulated as described in A and quantitative real-time RT-PCR was performed. *HERC5* expression levels are normalized to β_2-microglobulin expression. Changes in *HERC5* mRNA were significant over time. (D) Comparison of *HERC5* expression levels as determined by quantitative real-time RT-PCR in HSMECs, HUVECs and HSMCs.

LPS-stimulated ECs (Wrighton et al., 1996). We used this recombinant IκBα adenovirus to investigate the influence of NF-κB on *HERC5* gene expression. We found that overexpression of IκBα strongly reduces *HERC5* mRNA expression in LPS-activated HSMECs suggesting a crucial role of NF-κB in *HERC5* gene regulation (Fig. 3B). Similar results were obtained in LPS-stimulated HUMECs when *HERC5* mRNA expression levels were quantified by real-time RT-PCR (Fig. 3C).

To further characterize the NF-κB dependency of *HERC5* regulation, we cloned the putative *HERC5* promoter region of 1569 bp upstream of the cDNA start site into luciferase expressing reporter constructs. Bioinformatics analysis of this region revealed several sites for inflammation-related DNA-binding proteins, but no NF-κB consensus site was found. In reporter assays, basal promoter activity was barely detectable in HEK293 cells. In primary ECs neither basal nor inducible promoter activity was seen (data not shown). These data indicate that NF-κB-dependent upregulation of *HERC5* mRNA is probably not mediated via the proximal *HERC5* promoter up to –1569 bp.

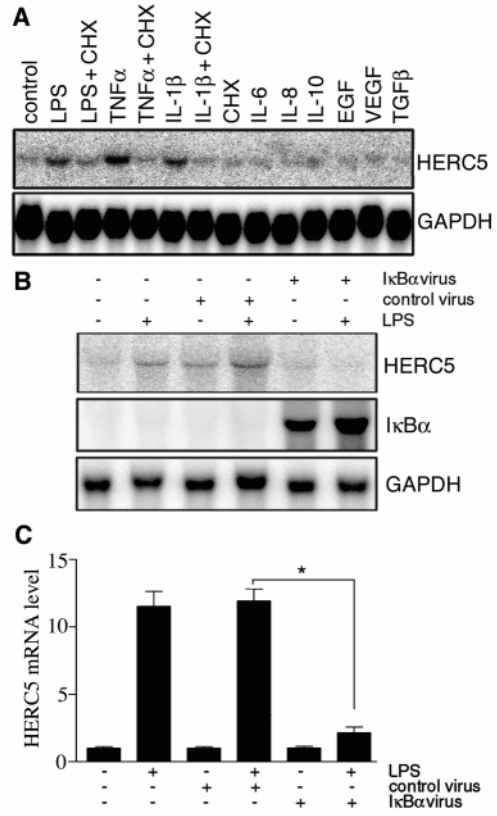

Fig. 3. Regulation of *HERC5* mRNA expression. (A) *HERC5* gene expression in HM2 cells in response to different stimuli after 8 hours of treatment. 10 μg of total RNA were loaded per lane for analysis. Northern blots were probed for *HERC5* expression and reprobed for *GAPDH* to confirm equal loading of RNA. CHX, cycloheximide; EGF, epidermal growth factor; IL, interleukin; LPS, lipopolysaccharide; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α, VEGF, vascular endothelial growth factor. (B) Northern blot analysis of rAd.IκBα-infected cells. HSMEC were infected with either the rAd. IκBα or a control adenovirus as indicated above each lane and 48 hours post infection cells were left unstimulated or stimulated with LPS for 6 hours. 10 μg of total RNA were analyzed for mRNA of *HERC5*, *IκBα* and *GAPDH* by subsequent hybridizations. (C) HUMECs were treated as described in B but stimulated with LPS for 8 hours; isolated RNA was analyzed by quantitative real-time RT-PCR. **P*<0.05.

Control of HERC5 protein levels

In order to determine whether *HERC5* mRNA regulation by inflammatory stimuli in ECs is reflected at the protein level, we raised a rabbit polyclonal antiserum against the C-terminal HECT domain of HERC5. Immunoprecipitation was performed from cell lysates of metabolically labeled vascular cells to identify endogenous levels of HERC5 protein. The HERC5 protein level in quiescent HSMECs was higher than in HUVECs and HSMCs (Fig. 4A), consistent with the respective mRNA levels (Fig. 2D). In contrast to mRNA upregulation, HERC5 protein is not increased in HSMECs and HUMECs upon LPS stimulation for 12-17 hours (Fig. 4B and data not shown). These results prompted us to analyze HERC5 protein turnover in LPS-treated microvascular ECs. Results from pulse-chase experiments in HSMECs and HUMECs revealed a half-life of 9 hours in the absence and of 2 hours

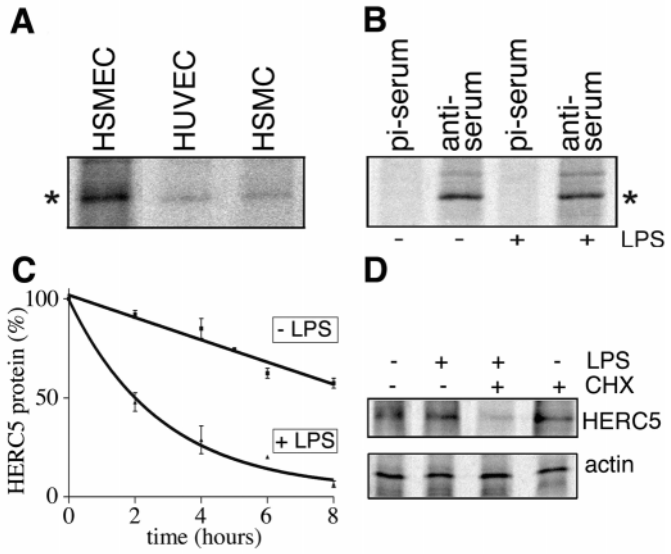

Fig. 4. Expression of HERC5 protein. (A) HERC5 protein expression. Immunoprecipitation with anti-HERC5 serum from HSMECs, HUVECs and HSMCs metabolically labeled for 16 hours were analyzed by 9% SDS-PAGE. Samples are normalized to cell number. HERC5 protein is marked by an asterisk. (B) Immunoprecipitation with anti-HERC5 serum from HSMECs metabolically labeled for 16 hours in the absence or presence of LPS were resolved by 6.5% SDS-PAGE. Duplicates were immunoprecipitated with pre-immune serum as controls. Integrity of ECs was tested by IL-8 secretion upon proinflammatory cytokine treatment before assaying. Cytosolic cell extracts are normalized to counts. HERC5 signal is marked by an asterisk. pi-serum, pre-immune serum. (C) Results from pulse-chase experiments in HSMECs in the absence (■) or presence (▲) of LPS during chase. The calculated regression curves were significantly different to each other ($P<0.05$). (D) HERC5 protein was precipitated from cell lysates of HSMECs metabolically labeled for 20 hours. Where indicated, LPS was added for 8 hours and CHX for 4 hours before cell lysis. As a control, actin was precipitated from the same protein extracts. CHX, cycloheximide.

in the presence of LPS (Fig. 4C and data not shown). From these data we conclude accelerated HERC5 protein turnover in ECs upon LPS stimulation. To prove that HERC5 protein detected in ECs activated with LPS for 16 hours results from de novo protein synthesis we used CHX to block HERC5 protein synthesis. Cells were metabolically labeled for 20 hours in total, LPS was added at 8 hours, and CHX at 4 hours before lysis. CHX was added 4 hours after LPS stimulation in order not to interfere with *HERC5* mRNA upregulation (validated in replicate experiments, data not shown). As shown in Fig. 4D, HERC5 protein level is restored in cells treated with LPS for 8 hours, but not in cells treated with CHX for 4 hours before analysis.

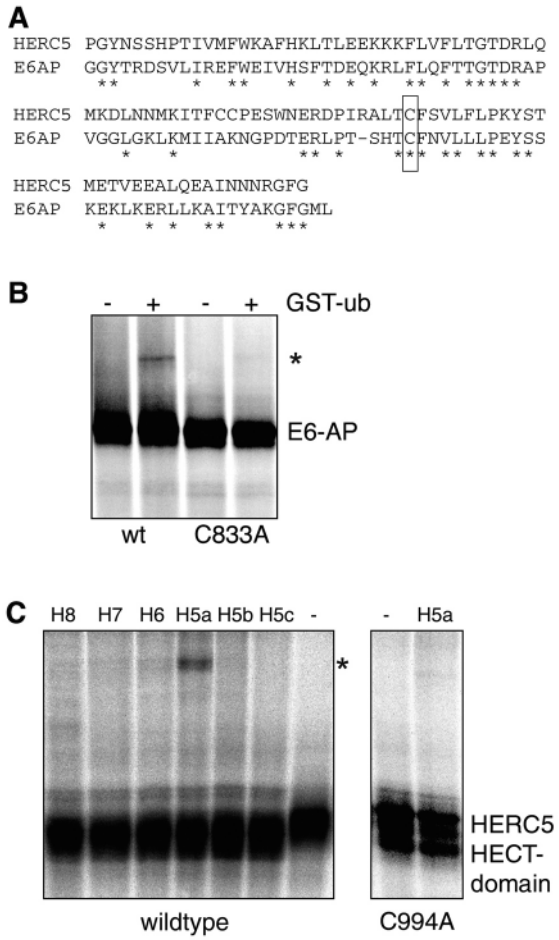

Fig. 5. In vitro thioester bond formation assay. (A) Comparison of the C-terminal amino acid sequences of E6-AP and HERC5 are shown. The catalytic site cysteine residues are boxed. Identical residues are marked by asterisks. (B) Wild-type or C833A mutant E6-AP proteins were synthesized in vitro in the presence of L-[^{35}S]methionine and tested for thioester bond formation in the presence (+) or absence (–) of GST-ubiquitin. Reactions were stopped in non-reducing sample buffer for 20 minutes at room temperature and analyzed by 9% SDS-PAGE and autoradiography. Slower migrating proteins are marked by an asterisk. GST-ub, GST-ubiquitin; wt, wildtype. (C) HERC5-HECT or HERC5-HECT C994A proteins were synthesized and processed as in B. Crude bacterial lysates from cells overexpressing a particular E2 enzyme were added to the samples as indicated. Slower migrating proteins are marked by an asterisk.

E3 ubiquitin ligase activity of HERC5

The C-termini of HERC5 and E6-AP are homologous and both proteins contain a HECT-domain, characteristic of E3 ubiquitin ligases. In contrast to RING and U-box proteins, HECT domain E3 ubiquitin ligases transiently accept ubiquitin from an E2 enzyme and a thioester bond is formed between the C-terminal Gly76 of ubiquitin and the conserved active site cysteine residue of the ubiquitin ligase. The active site cysteine residue was identified in E6-AP and is necessary for both ubiquitin thioester formation and targeting of substrates (Huibregtse et al., 1995). The homology between the C-termini of HERC5 and E6-AP suggests ubiquitin ligase activity for HERC5 (Fig. 5A) and thioester bond formation was assayed in vitro (Huibregtse et al., 1995). HERC5, E6-AP and mutated forms containing cysteine-to-alanine substitutions (C-A) were synthesized in the rabbit reticulocyte lysate system (RRL). Thioester bonds between E3 proteins and GST-ubiquitin (molecular mass, 34 kDa) result in an increase in molecular weight under non-reducing conditions. RRL provides E1 and E2 enzyme activities necessary for activation and transfer of GST-ubiquitin (Huibregtse et al., 1995). In case of E6-AP (100 kDa), an additional band migrating with an apparent molecular mass of about 130 kDa was detected with the wild-type protein, but not with the C833A mutated form (Fig. 5B). No GST-ubiquitin was transferred to HERC5 under these experimental conditions, indicating that the E2 enzymes present in the RRL were not able to transfer ubiquitin to HERC5. Therefore, the E2 enzymes H3, H5a, H5b, H5c, H6, H7, H8 and H10 were added to the assay (Jensen et al., 1995; Kaiser et al., 1995; Kumar et al., 1997; Nuber et al., 1996; Plon et al., 1993; Scheffner et al., 1994; Townsley et al., 1997). E2 enzyme activity was controlled as described in Materials and Methods. Only UbcH5a was able to transfer GST-ubiquitin to the active site cysteine residue of HERC5 (Fig. 5C and data not shown). Substitution of this cysteine by alanine (HERC5 C994A) prohibits this molecular weight shift. These results demonstrate that HERC5 is an active E3 ubiquitin ligase.

Discussion

By using a differential screening technique to isolate novel genes regulated by inflammatory stimuli we have identified a ubiquitin ligase. The protein belongs to the HERC family as it contains both a HECT (homologous to E6-AP C-terminus) domain necessary for transfer of ubiquitin and RCC1 (regulator of chromosome condensation) repeats with putative GTP exchange activity and is termed HERC5 (Cruz et al., 2001). The cDNA obtained by us is similar to the *Ceb1* cDNA identified in a yeast two-hybrid screen for cyclin-E-binding proteins (Mitsui et al., 1999) and both represent *HERC5*. The minor differences in the two cDNA sequences might be due to different sources of RNA used for cDNA synthesis or due to errors introduced by both reverse transcription and PCR. Three additional HERC proteins are known so far. HERC1 and HERC2 are closely related, giant proteins of more than 500 kDa and are both suggested to play a role in vesicular trafficking (Lehman et al., 1998; Rosa and Barbacid, 1997; Rosa et al., 1996). *HERC3* is a ubiquitously expressed gene and the encoded protein is located both in the cytosol and in vesicular-like structures (Cruz et al., 2001). Although all HERC family members contain a HECT domain, ubiquitin

ligase activity was hitherto shown only for HERC3 (Cruz et al., 2001; Huibregtse et al., 1995). The E2 enzymes UbcH5a and UbcH7 have been reported to transfer ubiquitin to HECT domain proteins; in fact, UbcH7 is used by HERC3 (Schwarz et al., 1998). We have identified UbcH5a as the only E2 enzyme capable of transferring ubiquitin to HERC5. With respect to the putative guanine exchange factor (GEF) activity of the RCC1 repeats in HERCs, GEF activity was shown only for HERC1 on ARF1, Rab3A and Rab5, consistent with its vesicular localization (Rosa et al., 1996). For HERC3 such a function was not detectable (Cruz et al., 2001). HERC5 does not localize to cytosolic vesicles (Mitsui et al., 1999) (data not shown) and therefore, a role as GEF for Rab and Rho GTPases is unlikely.

We found that expression of *HERC5* is rather restricted. High expression is only detectable in testis, consistent with the expression of several E2 ubiquitin-conjugating enzymes and other E3 ubiquitin ligases of the HECT family proteins (EDD and E6-AP) in this tissue (Baarends et al., 2000; Wong et al., 2002). During spermatogenesis, the ubiquitin system is required specifically for massive breakdown of proteins (Baarends et al., 2000). In all other tissues, *HERC5* mRNA expression levels are very weak or not detectable. We identified *HERC5* as an inflammatory-stimuli-dependent gene in primary microvascular ECs. Whereas basal expression of *HERC5* was hardly detectable in any of the vascular cells, *HERC5* upregulation followed a specific time course in both TNFα- or LPS-stimulated microvascular ECs, where *HERC5* mRNA levels started to increase after 4 hours and peaked at 9-16 hours. These results characterize HERC5 as a late inflammatory response gene and are consistent with de novo protein synthesis required for *HERC5* mRNA upregulation. The upregulation was restricted to IL-1β, TNFα and LPS in ECs, as no regulation was seen by stimulation of ECs with other cytokines such as IL-10, IL-8, IL-6, TGFβ, or growth factors such as VEGF and EGF. Cytokines stimulating *HERC5* mRNA expression activate cells via the NF-κB pathway. In fact, *HERC5* upregulation was also strongly reduced when NF-κB was inhibited. The NF-κB effect, however, is likely to be indirect not only because *HERC5* is a late response gene and protein synthesis is required for upregulation of mRNA, but also because no NF-κB consensus site could be identified yet. The absent promoter activity of reporter constructs in ECs indicates either a role of upstream promoter/enhancer elements or a substantial contribution of mRNA stability to *HERC5* mRNA upregulation. Although no pattern for regulation of mRNA stability was found by bioinformatics analysis (UTRScan), such a possibility cannot be excluded and a regulation of mRNA stability is common in inflammatory gene expression (Kracht and Saklatvala, 2002).

Importantly, the inflammatory-cytokine-induced increase in mRNA levels did not result in increased protein levels. Instead, stimulation of ECs by LPS led to a shorter half-life of HERC5 protein. Hence, induced mRNA levels are necessary to restore but not to increase HERC5 protein. Induced mRNA expression levels, but no increase at the protein level is also observed for the RING ubiquitin ligase XIAP in ECs upon treatment with TNFα (Stehlik et al., 1998b). A tight control on protein level was shown for the HECT ubiquitin ligase E6-AP (Nuber et al., 1996). The long half-life of E6-AP is significantly decreased when it is ectopically overexpressed in H1299 cells, because high levels of E6-AP induce its own degradation. As E3 proteins provide substrate specificity, their tight regulation is necessary to ensure controlled substrate degradation. Although the substrate(s) of HERC5 are currently unknown, the regulation of HERC5 upon LPS stimulation suggests a specific role for this E3 enzyme during the inflammatory response of ECs. It is tempting to speculate that in the early phase of inflammation, HERC5 protein levels are decreased, allowing short-term substrate accumulation. At later time points increased mRNA levels result in recovered protein levels and ensure timely substrate recognition and degradation. Such a mechanism could amplify the initial inflammatory response and, by causing degradation at a later stage could contribute to the resolution of inflammation. In fact, protein degradation has been implied in the process of resolution (Majetschak et al., 2000); however, the mechanism responsible is not known. HERC5 being an active E3 ubiquitin ligase, with specific transcriptional and post-translational regulation in ECs, might be a good candidate for such a process.

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