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Transcriptional Mechanism of Protein Kinase C-Induced Isoform-Specific Expression of the Gene for Endothelin-Converting Enzyme-1in Human Endothelial Cells

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ABSTRACT

Isoform-specific expression of endothelin-converting enzyme (ECE)-1, the major big endothelin-processing enzyme, is controlled by alternative promoters. Signaling pathways and transcriptional mechanisms of ECE-1 mRNA expression are largely unknown. To investigate ECE-1 isoform expression after protein kinase C (PKC) activation, we used phorbol 12-myristate 13-acetate (PMA) to stimulate primary cultured human umbilical vein endothelial cells and the related EA.hy926 cell line. ECE-1a mRNA was up-regulated (approximately 3-fold), whereas mRNA of alternative isoforms (b, c, and d) was unchanged, which was confirmed on the protein level. PMA effects on mRNA expression were suppressed by the PKC inhibitors H-7 and Calphostin C. Because increased ECE-1a expression was preceded by induction of the transcription factor Ets-1, we performed gel shift assays and demonstrated specific DNA/protein interactions involving the ETS binding motif GGAA. Luciferase reporter assays showed that PMA induced ECE-1a promoter activity about 2.5-fold in EA.hy926 cells. Similarly, coexpression of Ets-1 protein resulted in a dose-dependent increase in ECE-1a promoter activity (more than 8-fold). Using gel shift assays and mutation analysis, we identified two tandemly arranged Ets-1 binding sites (EBS) at −638 and −658, respectively, that are involved in transcriptional activation of the ECE-1a promoter by PMA or Ets-1. Moreover, we also found evidence for binding of a transcriptional repressor to EBS −638. The inhibitor of mitogen-activated protein kinase kinase, PD98059, inhibited PMA effects on ECE-1a mRNA expression and promoter activity, respectively. Our results provide the first detailed analysis of signaling pathways and transcriptional mechanisms involved in isoform-specific *ECE-1* gene expression.

The biologically active endothelin (ET) peptides are released from their virtually inactive precursors, termed big endothelins, by endothelin-converting enzymes (ECEs) (Rubanyi and Polokoff, 1994). As of this writing, two ECEs, termed ECE-1 and ECE-2, which are transcribed from different genes, have been cloned and functionally characterized (Xu et al., 1994; Emoto and Yanagisawa, 1995). Structurally, both ECEs belong to the thermolysin subfamily of proteases, which also comprises neutral endopeptidase (NEP 24.11), Kell blood group protein, and PHEX (formerly termed PEX) (Turner and Tanzawa, 1997). Both ECEs represent type II membrane-bound, zinc-binding metalloproteases with large extracytoplasmic domains. Comparison of bovine ECE-1 and ECE-2 cDNA sequences revealed an overall sequence identity of 59%. In contrast to ECE-2, which is expressed mainly in neural tissues, ECE-1 shows a broader tissue expression pattern. Detailed expression studies in human tissues revealed that the vascular endothelium represents a major site of constitutive ECE-1 expression (Korth et al., 1999).

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ABBREVIATIONS: ET, endothelin; ECE, endothelin-converting enzyme; NEP, neutral endopeptidase; ETS, E26 transformation-specific; PMA, phorbol 12-myristate 13-acetate; HUVEC, human umbilical vein endothelial cell; PKC, protein kinase C; MAPKK, mitogen-activated protein kinase kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; bp, base pair(s); GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; ODN, oligodeoxynucleotide; DMSO, dimethyl sulfoxide; EBS, ETS binding site; kb, kilobase; VSMC, vascular smooth muscle cell.

The human *ECE-1* gene was initially reported to span over 68 kilobases and to comprise 19 exons (Valdenaire et al., 1995). Analysis of cloned cDNA sequences revealed that human ECE-1, but also the rat homolog, is expressed in four mRNA isoforms, termed a (alternative nomenclature: β), b (α), c, and d, respectively, which differ exclusively in their 5′ termini (Schmidt et al., 1994; Shimada et al., 1995; Schweizer et al., 1997; Valdenaire et al., 1999). Analysis of recently released genomic sequence databases combined with transcriptional start point mapping extended the human *ECE-1* locus to a region of at least 120 kilobases (Funke-Kaiser et al., 2000). Because all isoform-specific ECE-1 exons contain a putative translation initiation codon, it is generally assumed that ECE-1 is expressed in protein isoforms with variant N-termini, suggesting different subcellular localization of ECE-1 isoforms. Analysis of the subcellular distribution of ECE-1 isoforms showed that ECE-1a is preferentially localized to the cytoplasmic membrane, whereas ECE-1b is found mainly in intracellular compartments. ECE-1c and -1d display an intermediate distribution pattern (Schweizer et al., 1997; Valdenaire et al., 1999). Although knowledge of the regulation of ECE-1 expression is still limited, there are some reports on increased expression of ECE-1 in vascular disease models, such as experimental arteriosclerosis of rabbit blood vessels (Grantham et al., 1998) and during neointima formation induced by balloon catheter injury of the rat carotid artery (Wang et al., 1996; Minamino et al., 1997).

Functional promoter studies using reporter gene assays provided evidence that ECE-1 isoform expression is regulated by alternative promoters rather than by differential splicing (Orzechowski et al., 1997; Funke-Kaiser et al., 2000). Sequence analysis of the human alternative ECE-1 promoters (all of which lack classical TATA boxes) revealed remarkable differences in the composition of consensus sequences for transcription factor binding suggesting elaborate mechanisms of transcriptional regulation. In contrast to the promoters directing the expression of alternative ECE-1 isoforms (b, d, and c), the human and bovine promoters of ECE-1a are characterized by multiple consensus sequences for binding sites of transcription factors of the ETS family (Orzechowski et al., 1999).

ETS proteins constitute a large family of winged helix-loop-helix transcription factors that bind to DNA sequences containing the core motif GGA/T. The DNA-binding domain is conserved between different ETS proteins and also between species, including human, mouse, and *Drosophila melanogaster* (Sharrocks et al., 1997). In human disease and in experimental animal models, expression of Ets-1 has been associated with tumor cell invasion (Kitange et al., 1999), angiogenesis (Oda et al., 1999), and wound healing (Ito et al., 1998).

We previously reported a strong induction of Ets-1 mRNA expression in the human endothelial cell line EA.hy926 by the phorbol ester PMA (Orzechowski et al., 1998). In the same experiment, up-regulation of ECE-1a isoform mRNA expression was demonstrated that led us to the hypothesis that Ets-1 may be involved in transcriptional regulation of ECE-1 expression. These observations prompted us to analyze the transcriptional mechanisms by which PMA stimulation of human endothelial cells may lead to increased mRNA and protein expression of ECE-1a. Using gel shift assays and functional promoter studies, we performed a detailed investigation of a possible functional role of Ets-1 in transcriptional regulation of the human *ECE-1* gene.

Materials and Methods

Endothelial Cell Culture. Human umbilical cords were obtained from the Department of Obstetrics at the Benjamin Franklin Medical Center of the Freie Universität (Berlin, Germany). Primary cultures of human umbilical vein endothelial cells (HUVEC) were isolated according to standard procedures. Briefly, umbilical cord veins were rinsed with 0.9% NaCl and endothelial cells were detached enzymatically using trypsin solution (1.25% in PBS containing Ca^{2+} and Mg^{2+}) at 37°C for 45 min. After rinsing with PBS, endothelial cells were collected by centrifugation, resuspended in endothelial cell growth medium (PromoCell, Heidelberg, Germany) and split in a 1:3 ratio. Confluent cultures of HUVEC showed typical cobblestone appearance and were further characterized by positive immunostaining for von Willebrand factor and absence of smooth muscle α-actin staining.

The permanent human endothelial cell line EA.hy926 was cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, hypoxanthine aminopterin thymidine supplement, 1 mM sodium pyruvate, nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin and was routinely split in a 1:16 ratio once a week. Cell culture media and supplements were obtained from Biochrom (Berlin, Germany).

Stimulation Experiments and RNA Extraction. Before treatment with 10 ng/ml PMA (Sigma, Deisenhofen, Germany), confluent cultures of HUVEC (second passage) were incubated in endothelial cell basal medium (PromoCell) for 24 h. EA.hy926 cells were incubated in medium containing 0.25% fetal calf serum for 48 h. To inhibit activation of PKC or mitogen-activated protein kinase kinase (MAPKK), EA.hy926 cells were pretreated with staurosporine (Sigma), H-7 (Sigma), Calphostin C (Sigma), or PD98059 (Calbiochem-Novabiochem, Bad Soden, Germany), respectively, for 60 min, before PMA was added. Total RNA was extracted using RNeasy kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Ten micrograms (HUVEC) or 20 μg (EA.hy926) of total RNA per lane was separated on 1% agarose formaldehyde gels and blotted on Hybond N membranes (Amersham Pharmacia Biotech Europe, Freiburg, Germany).

Probe Synthesis and Northern Blot Hybridization. Probes for Northern blot hybridization were synthesized by reverse transcription-PCR using Superscript reverse transcriptase (Invitrogen, Karlsruhe, Germany) and 5 μg of HUVEC total RNA. Gene-specific primers and fragment sizes were as follows: ECE-1a sense, 5′-ACAGGAGGCAGCCCTGATGC; antisense, 5′-CACCTGCAGGGAAGGAGGC; 121 bp. ECE-1 (bcd) sense, 5′-ATGTCGACGTACAAGCGGGCC; antisense, 5′-CTGCAGGCCGTTGGGGTATGC; 81 bp. Ets-1 sense, 5′-CTCCTGGCACCATGAAGGCG; antisense, 5′-GCAGTCTTTACCCAGGGCGC; 347 bp. GAPDH sense, 5′-CATTGACCTCAACTACATGG; antisense, 5′-TGGACTGTGGTCATGAGT; 427 bp. Amplification products were subcloned in the pCR2.1 vector (Invitrogen, Groningen, The Netherlands) using the TA cloning kit (Invitrogen). Insert identity was confirmed by sequencing using the Prism 377 DNA sequencer (Applied Biosystems, Rodgau-Jügesheim, Germany). Northern probes for Ets-1 and GAPDH were labeled using [^{32}P]dCTP (3000 Ci/mmol) and Rediprime (Amersham Pharmacia Biotech). Northern probes for ECE-1a and ECE-1(bcd) were labeled by radioactive PCR using 100 pg of the corresponding isoform-specific cDNA plasmid, 0.5 mM sense and antisense primer, 6 μl of [^{32}P]dCTP (3000 Ci/mmol), and 10 μM dATP, dGTP, and dTTP, in a final volume of 20 μl. Hybridization was carried out at 42°C overnight in a buffer containing 5× standard saline/phosphate/EDTA, 5× Denhardt's solution, 1% SDS, 50% formamide, and 100 μg/ml denatured salmon sperm DNA. After final posthybridization washes in 0.1× standard saline citrate/0.1% SDS, blots were exposed to Fuji BAS-MP 2040S image plates (Raytest Isotopenmeßgeräte, Straubenhardt, Germany).

hardt, Germany) for quantitative signal analysis in a Fuji BAS-1500 image plate scanner (Raytest). Subsequently, blot membranes were autoradiographed by exposure to XAR-5 X-ray films (Kodak, Stuttgart, Germany) at −20°C.

Generation and Characterization of the Antibody Specific for ECE-1a. A polyclonal isoform-specific antibody against ECE-1a protein was generated by immunization of New Zealand White rabbits using a synthetic N-terminal peptide KRGPGLTSSPPL (PolyPeptide Laboratories, Torrance, CA) coupled to the carrier protein KLH (Sigma). The immunization protocol was as follows: one s.c. injection of 200 μg of peptide in complete Freund's adjuvant (Sigma), followed by two s.c. injections of 100 μg of peptide in incomplete Freund's adjuvant, and three s.c. injections of 100 μg of peptide in PBS. Antibody titer as determined by enzyme-linked immunosorbent assay finally reached $1{:}10^6$. Antibodies were purified by protein A affinity chromatography (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Constructs expressing ECE-1 isoforms a, b, and c were generated by cloning the corresponding isoform-specific reverse transcription-PCR products in the expression vector pcDNA3 (Invitrogen). Isoform-specific ECE-1 sense primers and the common anti-sense primer were based on the previously published human ECE-1 cDNA sequences (GenBank/EMBL X91922, X91923, X98272, NM_001397). Specificity of the polyclonal anti-human ECE-1a antibody was tested by Western blot analysis. Ten micrograms of total protein of CHO cells, stable or transiently transfected with plasmids expressing human ECE-1a, -b, and -c, respectively, was separated by SDS-PAGE (4–12%) under nonreducing conditions and hybridized as described previously (Subkowski et al., 1998). Antigen was detected using streptavidin-alkaline phosphatase complex (Roche Molecular Biochemicals, Mannheim, Germany) and nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl phosphate substrate (Roche Molecular Biochemicals).

Protein Extraction. For membrane protein extraction, cells were scraped in ice-cold PBS after stimulation, pelleted, and homogenized using an Ultra-Turrax homogenizer (IKA-Labortechnik, Staufen, Germany) in 10 volumes of homogenization buffer (20 mM Tris-Cl, pH 7.4, 5 mM $MgCl_2$) containing the protease inhibitor cocktail Complete (Roche Molecular Diagnostics). After centrifugation at 1,000*g* to remove debris, crude membranes were pelleted by centrifugation at 100,000*g* for 45 min. To dissolve membrane proteins, the pellet was solubilized in homogenization buffer containing 1% Triton X-100. After centrifugation at 100,000*g* for 60 min, protein concentration was determined in the supernatant using DC Protein Assay (Bio-Rad, München, Germany). Membrane protein aliquots were stored at −80°C until used.

To extract nuclear proteins, 2×10^7 endothelial cells (HUVEC and EA.hy926) were stimulated with 10 ng/ml PMA for 6 h, scraped in 2.5 ml of ice-cold PBS, pelleted, and homogenized in hypotonic buffer [10 mM HEPES-KOH, pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, and Complete (Roche Molecular Biochemicals)]. Cells were incubated on ice for 20 min and briefly vortexed after adding 10% (v/v) IGEPAL-630 (Sigma). After centrifugation, the pellet was resuspended in 0.5 ml of hypertonic buffer [20 mM HEPES-KOH, pH 7.9, 25% (v/v) glycerol, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, Complete] and incubated on ice for 20 min with shaking. After centrifugation at 13,000*g*, nuclear protein concentration in the supernatant was determined using DC Protein Assay (Bio-Rad Laboratories), and aliquots were stored as described.

Western Blot Analysis. Twenty micrograms of protein was separated by 10% SDS-PAGE (acrylamide/bisacrylamide, 30:0.8) according to standard procedures and transferred to Protran nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany) by semidry blot transfer. Before blotting, gels were stained with Poinceau S dye to confirm equal protein loading. For detection of ECE-1a and ECE-1(bcd) protein, blots were hybridized overnight at 4°C with appropriate isoform-specific anti-ECE-1 antibody diluted 1:300. For detection of Ets-1 protein, blots were hybridized with polyclonal anti-Ets-1 antibody (sc-350X; Santa Cruz Biotechnology, Heidelberg, Germany) diluted 1:4000. Subsequently, blots were incubated with secondary anti-rabbit horseradish peroxidase antibody (Dianova, Hamburg, Germany). Signals were detected using enhanced chemiluminescence reagent (ECL; Amersham Pharmacia Biotech) and exposed to Hyperfilm-ECL (Amersham Pharmacia Biotech) autoradiography film.

Electrophoretic Mobility Shift Assay. End-labeling of Ets-1 consensus double-stranded oligodeoxynucleotides (ODN) was performed using T4 polynucleotide kinase (New England Biolabs, Frankfurt, Germany), 1 pmol of double-stranded ODN, and 40 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol). Binding reactions were carried out at room temperature for 30 min in a final volume of 25 μl (100 mM KCl, 15 mM HEPES-KOH, pH 7.5) using 10 μg of nuclear protein, 1 μg of poly d[(A-T)] (Roche Molecular Biochemicals), 1 μg of poly d[(I-C)], and 20,000 cpm end-labeled Ets-1 consensus ODN. Competitive binding assays were performed with excess amounts of unlabeled double-stranded wild-type (core motif GGAA) or mutated (core motif GGCA) Ets-1 consensus ODN (sequences according to Hultgardh-Nilsson et al., 1996). For EMSA, either 2 μg of the polyclonal anti-Ets-1 antibody (sc-350X) or 5 μl of anti-Ets-1 antiserum (generously provided by Dr. James Hagman) was added. The latter antibody, which showed much better EMSA reactivity, was used to investigate Ets-1 binding to promoter sites −638 and −658, respectively. Binding reactions were electrophoresed at 7.5 V/cm in 4% polyacrylamide gels in Tris-glycine buffer. After vacuum drying, gels were autoradiographed as described.

Expression of Recombinant Ets-1 Protein. A full-length cDNA of human Ets-1 was generated by reverse transcription-PCR of EA.hy926 total RNA. Ets-1 primer sequences corresponded to nucleotide positions 116 and 1448 of the published human Ets-1 cDNA sequence (GenBank/EMBL J04101): sense, 5′-CACCATGAAGGCGGCCGT; antisense, 5′-CCATCACTCGTCGGCATCT (antisense). The amplification product was subcloned in the expression vector pCR3.1 (Invitrogen) and sequenced to confirm identity and correct orientation. For expression of recombinant Ets-1 protein in vitro, TNT T7 Quick Coupled Transcription/Translation System kit (Promega, Mannheim, Germany) was used exactly as recommended by the manufacturer's protocol.

Transfection Procedures and Reporter Gene Assays. Twenty-four hours before transfection, about 100,000 EA.hy926 cells per well were seeded in 12-well plates. Generation of ECE-1a promoter luciferase constructs based on the firefly (*Photinus pyralis*) luciferase vector pGL3basic (Promega) was reported previously (Orzechowski et al., 1997). Transfection was performed using 0.5 μg of firefly luciferase construct and 1.5 μl of Fugene 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's protocol. Cells were harvested 16 h after PMA stimulation. In Ets-1 coexpression experiments, 0.4 μg of ECE-1a promoter luciferase construct and 0.1 μg of Ets-1 expression construct were used. In all experiments, transfection efficiencies were controlled by cotransfection of 0.05 μg of *Renilla reniformis* plasmid RL-null (Promega). Firefly and *Renilla* luciferase activities were assayed in a Berthold Lumat LB9501 using the Dual-Luciferase Reporter Assay System (Promega).

Statistical Analysis. Means of the firefly/*R. reniformis* lociferase ratios were analyzed by two-tailed Student's t test for unpaired samples. Significance was accepted when p was < 0.05.

Results

Effect of PMA Stimulation on mRNA Expression of ECE-1 Isoforms and of the Transcription Factor Ets-1 in Cultured Human Endothelial Cells. The temporal mRNA expression pattern of ECE-1 isoforms in HUVEC treated with 10 ng/ml PMA was analyzed by Northern blot (Fig. 1A). Using a probe specific for the ECE-1a isoform, we found 3.5-fold increased intensities (at 12 and 24 h, respec-

tively) of two bands, which corresponded to the two known ECE-1 transcripts of 4.8 and 3 kb, respectively. In contrast, rehybridization of the membrane with a probe specific for ECE-1(bcd) mRNA resulted in nearly constant signal intensities (less than 25% difference compared with controls). Analyzing the expression of Ets-1 mRNA, we detected approximately 7-fold increased hybridization signals at 3 h, which corresponded to the previously reported human Ets-1 transcripts of 6.8 kb and 2.7 kb, respectively. Essentially identical Northern blot results were obtained analyzing PMA-stimulated EA.hy926 cells (Fig. 1B), showing 3- and 7-fold increased mRNA levels of ECE-1a and Ets-1, respectively. Specificity of PKC activation by PMA was confirmed in EA.hy926 cells by pretreatment with protein kinase C inhibitors, staurosporine, H-7, and Calphostin C, respectively. The very potent but nonselective PKC inhibitor staurosporine completely inhibited induction of Ets-1 and of ECE-1a mRNA expression at 100 nM concentration (data not shown). H-7 and the highly specific inhibitor Calphostin C showed total suppression of PMA effects on ECE-1a mRNA expression at 12 h (10 μM and 100 nM, respectively), and partial suppression of PMA-induced Ets-1 mRNA levels at 3 h (Fig. 2).

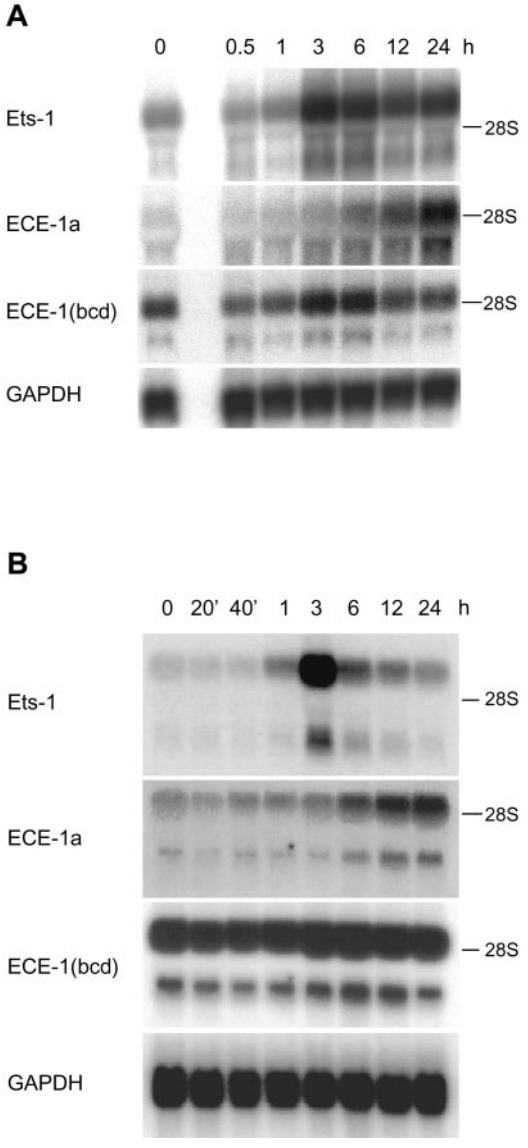

Fig. 1. Expression of ECE-1 mRNA isoforms in PMA-stimulated human endothelial cells. Confluent cultures of HUVEC (A) and EA.hy926 cells (B) were stimulated with 10 ng/ml PMA. Twenty micrograms of total RNA was isolated at the time points indicated, separated in formaldehyde-agarose gels, and analyzed by Northern blot as described under *Materials and Methods*. Differential analysis of ECE-1 isoform expression was performed using hybridization probes specific for ECE-1a and for the alternative isoforms ECE-1(bcd), respectively. GAPDH hybridization served as control for RNA load.

Characterization of Polyclonal Antibody Directed against ECE-1a. To confirm PMA-induced increases in ECE-1a mRNA expression on the protein level as well, we first generated a polyclonal antibody targeted at the N-terminal peptide of human ECE-1a. Cross-reactivity with alternative ECE-1 isoforms was excluded by analysis of CHO cells transiently and stably transfected with recombinant plasmids expressing human ECE-1a, -b, and -c, respectively (Fig. 3). After transient transfection of the ECE-1a expression plasmid, only one band of the expected size of approximately 250 kDa was detected under nonreducing conditions using the ECE-1a–specific antibody (Fig. 3, blot A) or the monoclonal antibody E15/6, which detects all ECE-1 isoforms (Fig. 3, blot B). Extracts of CHO cells expressing ECE-1b or ECE-1c did not show reactivity to the ECE-1a antibody but were reactive with the monoclonal antibody. In stably transfected cells, we detected two equally intense bands of 250 kDa and a second signal of slightly more than 250 kDa (Fig. 3, blot C). Apparently, both signals represented ECE-1a because preadsorption of the polyclonal antibody with the ECE-1a peptide eliminated both bands (not shown).

Effect of PMA Stimulation on Protein Expression of the Transcription Factor Ets-1 and ECE-1 Isoforms in Human Endothelial Cells. After RNA expression analysis, we first studied Ets-1 protein expression in PMA-stimulated EA.hy926 cells and found a strongly increased Ets-1 protein level at 6 h. As expected, clearly higher levels of Ets-1 protein were detected in nuclear extracts compared with the cytosolic fraction (Fig. 4A, upper blot). In agreement with previously published literature (Koizumi et al., 1990), we detected at least three Ets-1 isoforms in the nuclear fractions of EA.hy926 cells corresponding to reported molecular mass isoforms ranging from 39 kDa to 48 kDa/52 kDa. Similarly, we found highly augmented levels of Ets-1 protein variants corresponding to isoforms of 39 kDa to 48 kDa/52 kDa in the

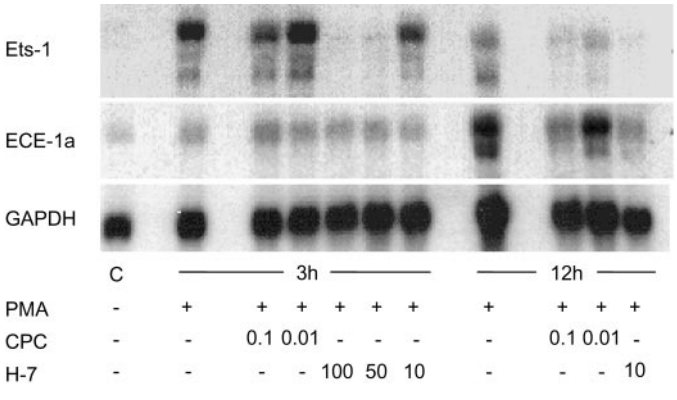

Fig. 2. PKC inhibition experiments. EA.hy926 cells were stimulated with 10 ng/ml PMA in the presence of the PKC inhibitors Calphostin C (CPC) or H-7. Ets-1 and ECE-1a mRNA expression at indicated time points was analyzed by Northern blot. Inhibitor concentrations (micromolar) are indicated. GAPDH hybridization served as control for RNA load.

nuclear fraction of PMA-stimulated HUVEC showing maximum expression levels at 6 and 12 h, respectively (Fig. 4A, lower blot).

We then studied the protein expression of isoforms ECE-1a and ECE-1(bcd) in PMA-stimulated EA.hy926 cells. Compared with DMSO-treated control cells, we detected clearly increased protein levels of ECE-1a at 12 and 24 h, respectively, in PMA-stimulated cells using the polyclonal rabbit anti-ECE-1a antibody (Fig. 4B, upper blot). Corresponding to the results obtained in stably transfected CHO cells (Fig. 3), the anti-ECE-1a antibody yielded two bands, one at about 115 kDa and a second one of higher molecular mass. In contrast to ECE-1a expression, we did not detect any relevant changes in the protein levels of ECE-1(bcd) using a polyclonal antibody targeted against an epitope encoded by exon 2 that is shared by these isoforms (Fig. 4B, lower blot).

DNA Binding of Nuclear Proteins Expressed in PMA-Stimulated Human Endothelial Cells. PMA-induced DNA-protein interactions involving ETS proteins were analyzed by EMSA using Ets-1 consensus ODN. Nuclear extracts of PMA-stimulated HUVEC yielded one intense signal corresponding to a higher molecular mass complex and one less intense signal of lower molecular mass (Fig. 5A, lane 1). Specificity of protein-DNA complexes was studied by compet-

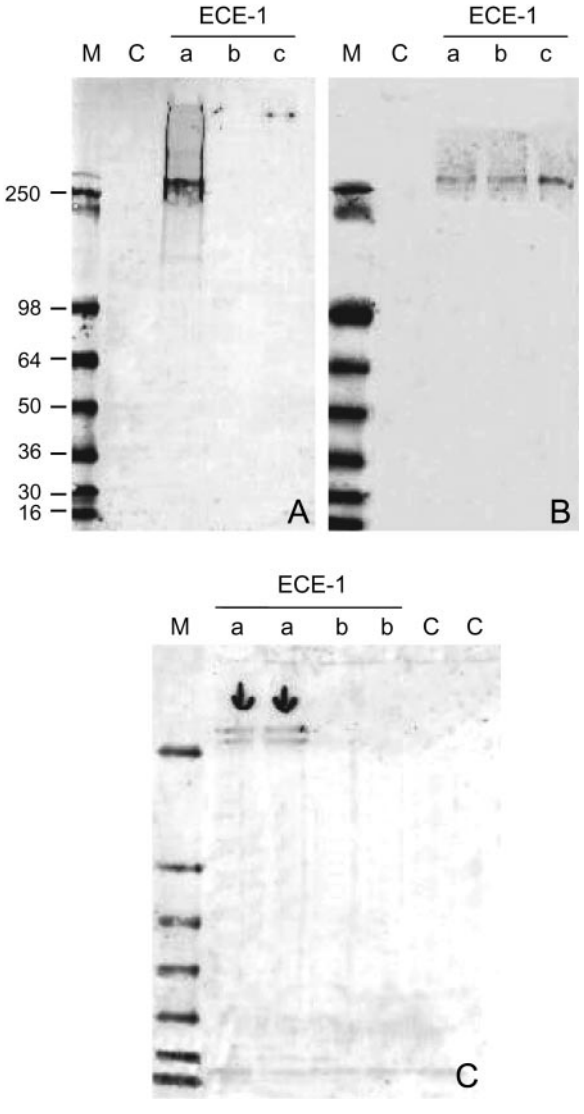

Fig. 3. Specificity of the polyclonal anti-ECE-1a antibody. Protein extracts of CHO cells transfected with expression constructs encoding human ECE-1 isoforms a, b, and c, respectively, were analyzed by Western blot. Blots of transiently transfected cells were hybridized with polyclonal antibody directed against the N-terminal peptide of human ECE-1a (A) or monoclonal antibody E15/6, which was raised against recombinant human ECE-1 protein and detects all isoforms (B). CHO cells stably transfected with constructs expressing ECE-1 isoforms a, b, and c, respectively, were hybridized with polyclonal anti-ECE-1a antibody (C). Ten micrograms of total protein was loaded per lane and separated by SDS-PAGE (4–10%) under nonreducing conditions. Molecular masses (in kilodaltons) of marker (M) proteins are indicated on the left side of the blots. C, untransfected control cells.

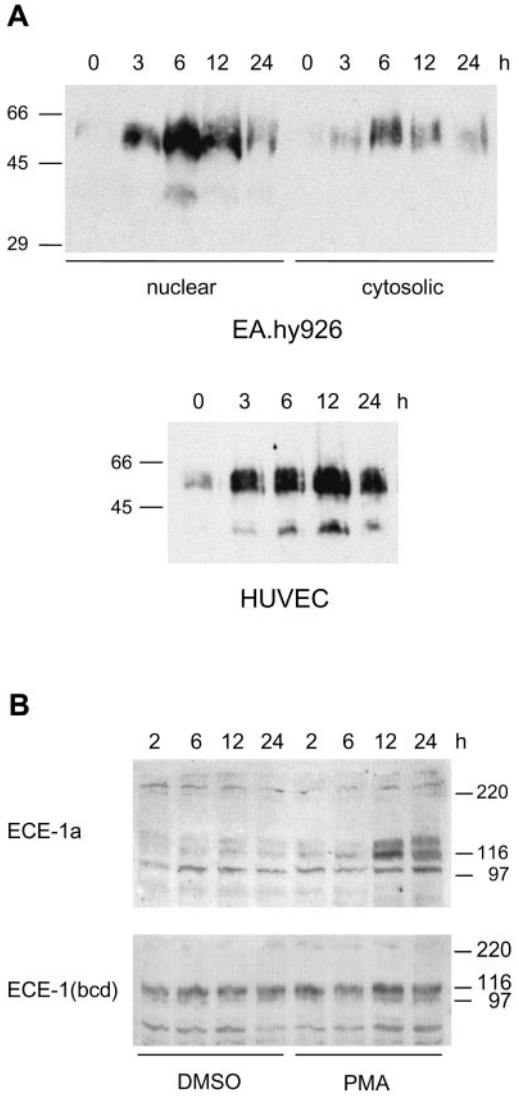

Fig. 4. Protein expression of Ets-1 and of ECE-1 isoforms in human endothelial cells stimulated with 10 ng/ml PMA. To detect Ets-1 protein in EA.hy926 cells (A, upper blot) and in HUVEC (A, lower blot), nuclear and cytosolic proteins were isolated at indicated time points as described under *Materials and Methods*. Twenty micrograms of protein per lane was separated under reducing conditions by SDS-PAGE and blots were hybridized with a polyclonal anti-Ets-1 antibody. To detect ECE-1 protein isoforms (B), membrane proteins of PMA-stimulated EA.hy926 cells and of DMSO-treated controls were isolated at indicated time points. Twenty micrograms of protein was separated by SDS-PAGE and hybridized with polyclonal antibodies directed against the N-terminal peptide of ECE-1a and a peptide sequence that is shared by the alternative isoforms ECE-1(bcd). Molecular masses (in kilodaltons) of marker proteins are indicated on the left.

itive binding assays, which showed complete inhibition of complex formation by a 100-fold molar excess of unlabeled Ets-1 binding ODN (Fig. 5A, lanes 2–4). Specificity was further confirmed by the observation that 100- and 1000-fold molar excesses of unlabeled mutated ODN did not suppress complex formation (Fig. 5A, lanes 5 and 6) and by failure of band shift using a mutated Ets-1 binding ODN (Fig. 5A, lane 8). Addition of a polyclonal anti-Ets-1 antibody resulted in a supershifted band that was accompanied by clearly decreased intensities of shifted bands (Fig. 5A, lane 7). With the exception of two equally intense band shift signals, essentially the same results were obtained with nuclear extracts of PMA-stimulated EA.hy926 cells (Fig. 5B).

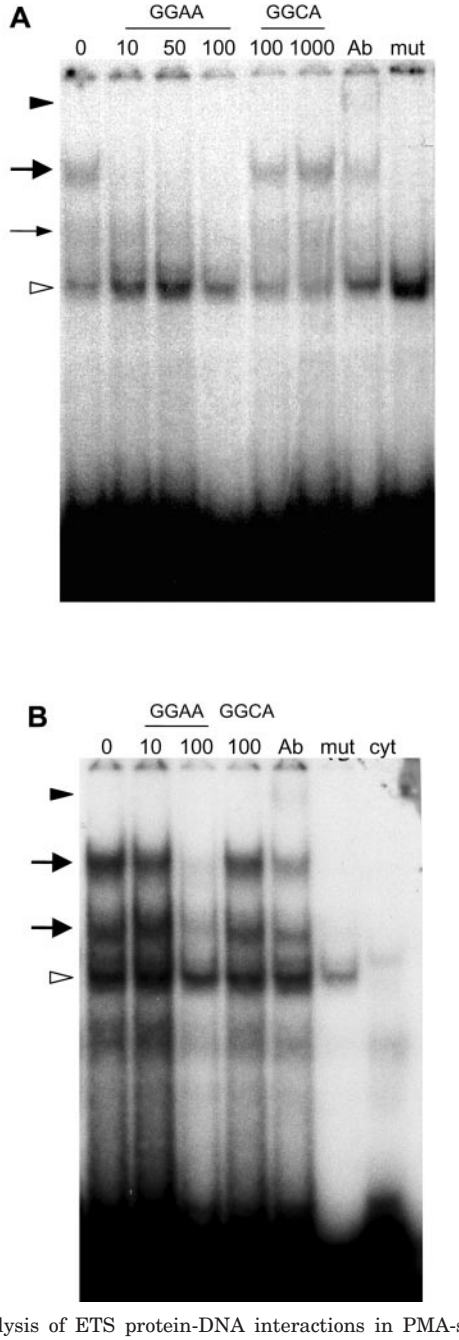

Fig. 5. Analysis of ETS protein-DNA interactions in PMA-stimulated human endothelial cells. HUVEC (blot A) and EA.hy926 cells (blot B) were stimulated with 10 ng/ml PMA and nuclear extracts were isolated after 6 h. EMSA was performed as described under *Materials and Methods* using ^{32}P-labeled ODN harboring a consensus sequence for Ets-1 binding (containing the core sequence GGAA) or a mutated sequence (core sequence GGCA). Increasing amounts (molar excess as indicated) of unlabeled consensus or mutant competitor ODN were added. Specific band shifts are indicated by arrows. The open arrowhead marks a nonspecific band shift. Addition of polyclonal anti-Ets-1 antibody (Ab) induced super shift (filled arrowhead) and partial suppression of shifted bands. Cytoplasmic protein (cyt) did not induce a band shift.

PMA and Ets-1 Coexpression Activates the ECE-1a Promoter. To further analyze whether PMA induced ECE-1a mRNA expression due to transcriptional activation, we investigated the effect of PMA on the activity of ECE-1a promoter construct −1,206 in transient transfection assays. Compared with DMSO controls, we found an increase in luciferase activity of about 2.5-fold in PMA-stimulated cells (Fig. 6A). To investigate whether Ets-1 coexpression would be able to *trans*-activate the ECE-1a promoter, we coexpressed Ets-1 and found up to 8-fold activation of the ECE-1a promoter compared with cells cotransfected with the control vector (Fig. 6B). Finally, we demonstrated by cotransfection of increasing amounts of Ets-1 expression plasmid that the degree of activation of the ECE-1a promoter was dependent on the Ets-1 coexpression level (Fig. 6C).

Identification of Putative Ets-1 Binding Regions in the ECE-1a Promoter. Analysis of the ECE-1a promoter using MatInspector sequence analysis software (http://genomatix.gsf.de/; Genomatix Software GmbH, Munich, Germany) identified 11 consensus sequences for binding of Ets-1 or other ETS family proteins (Fig. 7A). To localize the functional promoter regions with regard to activation by Ets-1, we cotransfected serial ECE-1a promoter deletion mutants with the Ets-1 expression construct and control plasmid pCR3.1, respectively (Fig. 7B). Whereas mutant −498 showed less than 2-fold activation by Ets-1 coexpression, constructs −736, −962, and −1206 were induced 6.5-fold, 9-fold, and 9.5-fold, respectively (Fig. 7C). These results indicated the presence of major activating Ets-1 binding sites in two ECE-1a promoter regions, ranging from −498 to −736 (containing tandemly arranged Ets-1 consensus sequences at −638 and at −658) and from −736 to −974 (containing tandemly arranged Ets-1 consensus sequences at −785 and at −814), respectively.

Because the strongest activating effect was associated with the promoter region containing Ets-1 consensus sequences at −638 and −658, respectively, we applied both sequences to EMSA. Using recombinant Ets-1 protein as binding partner, both ODNs displayed band shifts that were suppressed by an excess amount of the respective unlabeled ODN as well as supershifted bands when coincubated with anti-Ets-1 antiserum (Fig. 8). Corresponding EMSA using nuclear extracts of PMA-stimulated EA.hy926 cells yielded supershifted complexes of higher molecular mass, indicating binding of a nuclear coactivator protein to DNA-bound Ets-1.

EBS −638 Is a Major *cis*-Acting Element within the ECE-1a Promoter. Introduction of a point mutation into the core binding motif (GGA to GGC) of EBS −638 dramatically increased promoter activity under control conditions, indicating the binding of a transcriptional repressor to this *cis* element (Fig. 9A, open columns). In contrast, no change in promoter activity under control conditions was observed when consensus sequence −658 was mutated. Activation of the ECE-1a promoter by Ets-1 coexpression was completely abolished by mutation of EBS −638, and mutation at −658

reduced Ets-1 effects by approximately 30% (Fig. 9, A, filled columns, and B). Double mutation of EBS −638 and −658 showed essentially the same results as the single mutation of EBS −638. We finally addressed the question how the mutant promoter constructs may respond to PMA stimulation. Similar to Ets-1 coexpression, we found that mutation of EBS −638 decreased PMA activation by about 80%, whereas mutant −658 showed only a moderate reduction of PMA effects (Fig. 9C).

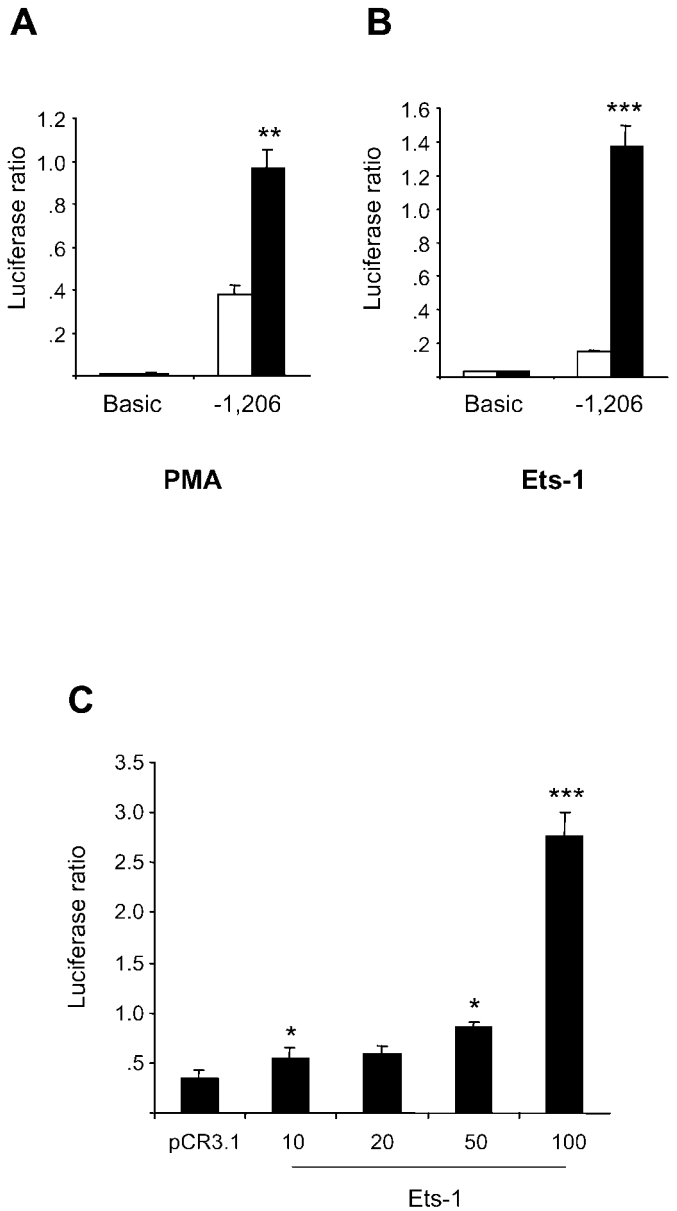

Fig. 6. Functional analysis of the human ECE-1a promoter. EA.hy926 cells were transiently transfected with ECE-1a promoter/firefly luciferase construct −1,206 and stimulated with PMA for 14 h (A, filled bars) or by coexpression of Ets-1 after cotransfection of 100 ng of expression plasmid/ml (B, filled bars). Treatment with DMSO (A) or cotransfection of pCR3.1 (B) served as negative control (open bars). A promoterless firefly luciferase construct (Basic) served as background control. The effect on promoter activity of increasing amounts of Ets-1 expression construct (indicated in ng/ml) is shown in C. *R. reniformis* plasmid RL-null was cotransfected for standardization, and promoter activities are expressed as firefly/*R. reniformis* luciferase ratios. Each bar represents the mean of $n = 4$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

MAPKK Is Activated Downstream of PKC and Mediates Transcriptional Activation of ECE-1a Expression. To analyze whether activation of the MAPKK (MEK1/2)-p44/

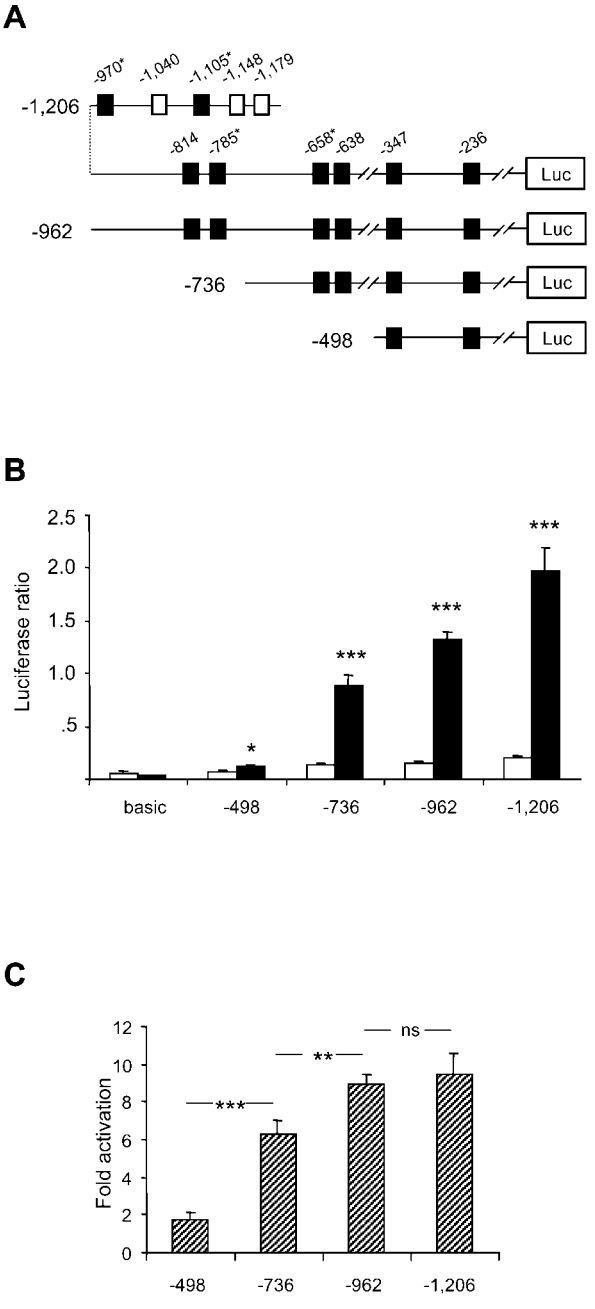

Fig. 7. A, analysis of putative ETS binding sites in serial deletion mutants of the human ECE-1a promoter cloned in luciferase reporter plasmid. The ECE-1a promoter sequence was analyzed using MatInspector software. ■, Ets-1 consensus sequences. □, other ETS consensus sequences. Positions of the ETS core motifs relative to the 3′ end of the promoter insert (position −1 = immediately upstream of the initiation start codon of human ECE-1a) are indicated. Asterisks indicate antisense orientation of the consensus sequence. Lengths of promoter deletion mutants are indicated on the left. Luc, luciferase. B, effect of Ets-1 coexpression (100 ng of expression construct/ml) on the activity of ECE-1a promoter deletion mutants. C, relative promoter activation by Ets-1 coexpression. Luciferase ratios measured under conditions of Ets-1 coexpression were standardized to the mean of the corresponding pCR3.1 control experiments. Promoter activities are expressed as firefly/*R. reniformis* luciferase ratios. Filled bars, Ets-1 coexpression. Open bars, pCR3.1 control. Each bar represents the mean of $n = 4$. *, $p < 0.05$, ***, $p < 0.001$.

p42 pathway downstream of PKC is involved in ECE-1a mRNA expression, we preincubated EA.hy926 cells with various concentrations of the MEK1/2 inhibitor, PD98059, before PMA stimulation and found a dose-dependent decrease in ECE-1a mRNA levels (Fig. 10A). The suppressing effect was detectable at inhibitor concentrations of 5 and 10 μM, respectively, and 50 μM PD98059 resulted in 80% reduction of PMA-induced ECE-1a mRNA levels (Fig. 10B). The Northern blot data were confirmed on the promoter level showing a dose-dependent reduction in reporter gene activities by PD98059 (Fig. 10C).

Discussion

In the present study, we have analyzed transcriptional and cellular signaling mechanisms involved in specific up-regulation of the ECE-1a isoform after PKC activation in cultured human endothelial cells. In primary cultured HUVEC and in the related cell line, EA.hy926, we demonstrated essentially identical temporal gene expression patterns of ECE-1 isoforms, which also holds true for the rapid and strong induction of de novo expression of the transcription factor Ets-1 on mRNA and protein levels, respectively. The temporal distance between induction of Ets-1 expression and increased expression of ECE-1a is in agreement with our previously postulated hypothesis that Ets-1 may be involved in transcriptional regulation of ECE-1a (Orzechowski et al., 1998), which is further underscored by the presence of multiple Ets-1 consensus sequences in the human ECE-1a promoter, some of them being evolutionarily conserved (Orzechowski et al., 1999).

Induction of ECE-1a mRNA expression by PMA was selective with regard to PKC, because we demonstrated that ECE-1a mRNA levels were efficiently suppressed by inhibitors of PKC. Regarding the regulatory function of PKC in human endothelial cells, the *ECE-1* gene has to be added to

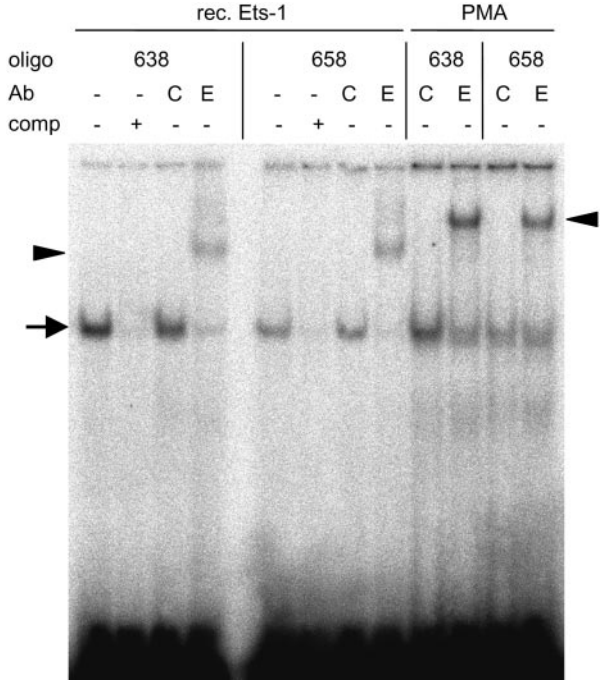

Fig. 8. Gel shift analysis of Ets-1 binding to consensus sequences −638 and −658, respectively. EMSA was performed using recombinant Ets-1 protein or nuclear extracts of PMA-stimulated EA.hy926 cells. Arrows, shifted bands; arrowheads, supershifted complexes. Ab, antibody; C, control antiserum; E, anti-Ets-1 antiserum; comp, competitor ODN. Signals were detected using a Fuji BAS-1500 image plate scanner.

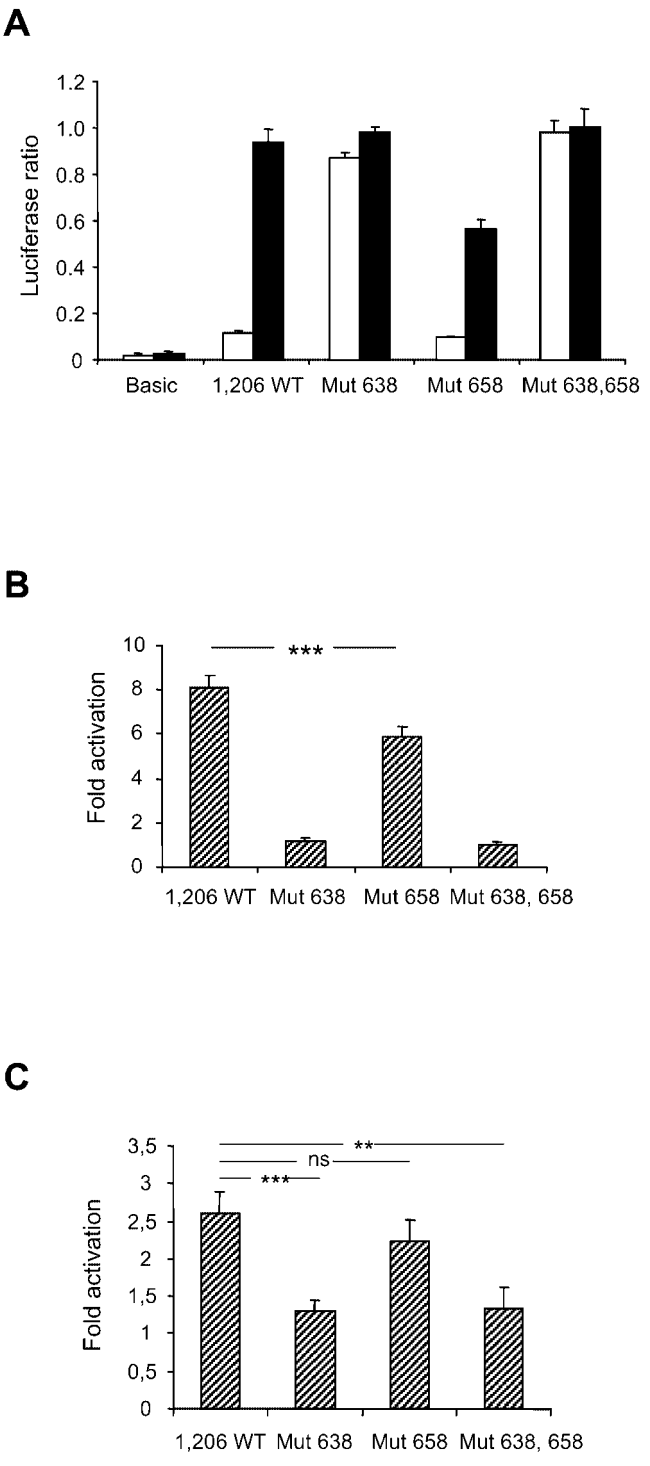

Fig. 9. Effect of point mutations of Ets-1 consensus sequences −638 and −658, respectively. A, promoter activities were measured under conditions of cotransfection of pCR3.1 vector (open columns) or Ets-1 coexpression (100 ng/ml; filled columns). B, relative promoter activation by Ets-1 coexpression. C, relative promoter activation by PMA stimulation. Promoter activities are expressed as firefly/*R. reniformis* luciferase ratios. Relative activation was calculated by standardization of luciferase ratios in stimulation experiments to the mean of the corresponding control experiment ($n = 4$). WT, wild type. **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

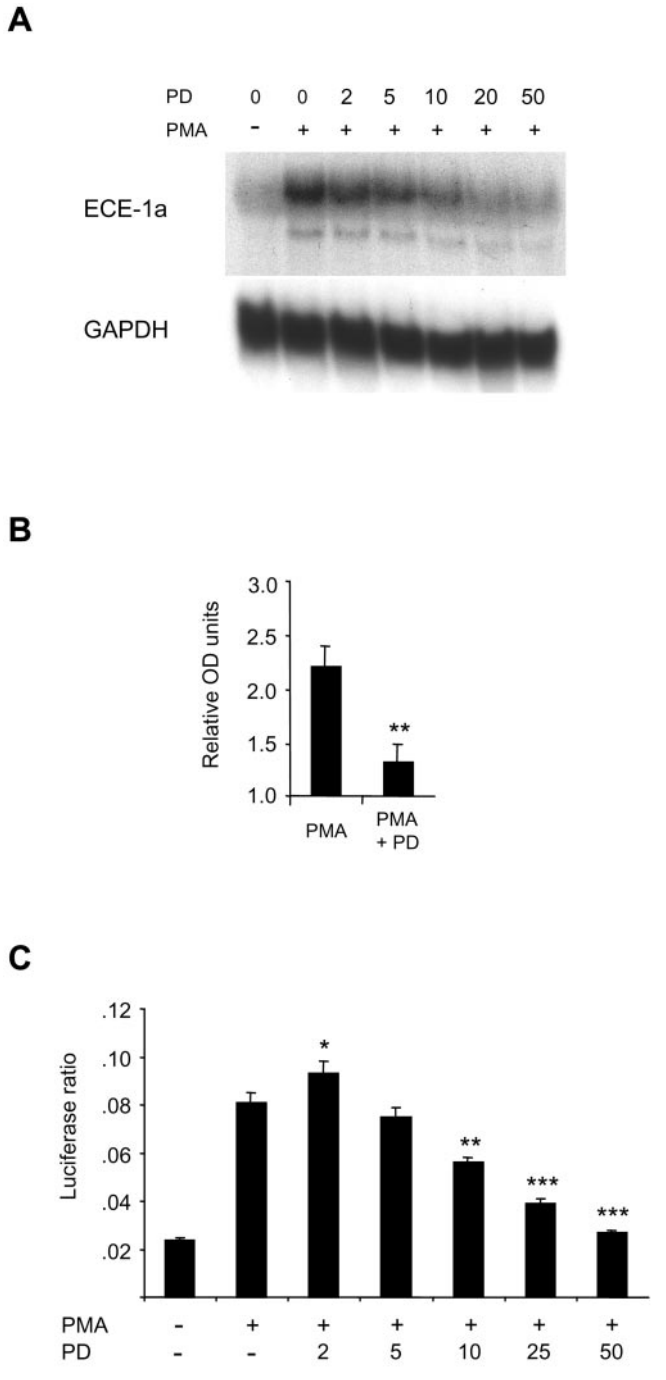

Fig. 10. Concentration-dependent effect of MEK inhibition on PMA-induced ECE-1a mRNA expression. A, EA.hy926 cells were preincubated with PD98059 for 60 min (μM concentrations as indicated) before stimulation with PMA for 24 h. Expression of ECE-1a mRNA was performed by Northern blot. C, DMSO-treated control cells. GAPDH expression served as control for RNA load. B, suppression of PMA-induced ECE-1a mRNA expression by 50 μM PD98059 (PD). RNA levels were quantified using a Fuji BAS-1500 image plate scanner. Individual ECE-1a mRNA signals were standardized against GAPDH signals. Signals of DMSO-treated controls were arbitrarily set as 100%. **, $p < 0.01$ ($n = 3$). C, Effect of PD98059 on PMA-stimulated ECE-1a promoter activity. EA.hy926 cells were transfected with ECE-1a promoter/firefly luciferase construct −1,206. Twenty-four hours after transfection, cells were incubated with increasing concentrations (μM as indicated) of PD98059 (PD) for 60 min, before stimulation with PMA was started. Promoter activities were determined after 24 h of PMA stimulation and are expressed as firefly/*R. reniformis* luciferase ratios. Each bar represents the mean of $n = 4$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

the group of PKC-activated genes that encode biosynthetic enzymes of vasoactive mediators, such as prostaglandin H synthase (Xu et al., 1996), endothelial constitutive nitric oxide synthase (Li et al., 1998), and angiotensin-converting enzyme (Villard et al., 1998).

Ets-1 protein expression in PMA-stimulated HUVEC and EA.hy926 cells was in agreement with results reported previously showing expression of several Ets-1 isoforms in the human T-cell line CEM that are due to alternative splicing of exon 7 (accounting for reduction in molecular mass of approximately 10 kDa) and phosphorylation (Koizumi et al., 1990). According to this work, high-molecular mass forms in our PMA-stimulated human endothelial cells probably correspond to p48 and p51 and/or to their phosphorylated isoforms p49 and p52, respectively. The low-molecular mass isoform, which is expressed on a low level, most likely corresponds to the 39-kDa isoform. PMA-induced up-regulation of Ets-1 expression, however, is not specific for endothelial cells inasmuch as it was also observed, for example, in vascular smooth muscle cells (VSMC) (Naito et al., 1998). We also confirmed PMA-induced changes in ECE-1 isoform mRNA expression on the protein level using specific polyclonal antibodies directed against an N-terminal peptide of ECE-1a and a peptide encoded by exon 2 of the *ECE-1* gene, which is shared by the alternative ECE-1 isoforms (b, c, and d). The functional significance of increased Ets-1 protein expression was further validated by EMSA using an ODN with consensus sequence for binding of Ets-1 (Hultgardh-Nilsson et al., 1996) and nuclear extracts of PMA-stimulated HUVEC or EA.hy926 cells. First, band shifts were efficiently inhibited by unlabeled competitor ODN. Second, the ODN mutated in the core ETS binding motif failed to induce specific band shifts. Third, excess of mutated ODN did not suppress band shifts. Finally, addition of anti-Ets-1 antibody to the binding reaction resulted in a supershifted band running in parallel with significantly decreased intensities of the shifted bands.

Additional evidence that PMA activates ECE-1a expression by a transcriptional mechanism via activation of the transcription factor Ets-1 was provided by our functional promoter studies. First, PMA treatment activates the ECE-1a promoter in transiently transfected EA.hy926 cells. Second, overexpression of Ets-1 strongly induces ECE-1a promoter activity in a dose-dependent manner. The promoter response obtained with increasing concentrations of Ets-1 expression plasmid showed a steep increase when a certain "threshold" amount of expression plasmid was cotransfected. This finding corresponds to the results of our PKC inhibition experiments showing complete suppression of the PMA-induced increase in ECE-1a mRNA expression at inhibitor concentrations that partially suppressed Ets-1 mRNA induction.

Analysis of ECE-1a promoter function by transfection of deletion mutants and Ets-1 coexpression identified two activating regions of 239 bp (upstream of −498) and 227 bp (upstream of −736), respectively. According to our supershift analyses, Ets-1 consensus sequences at −638 and −658 (which are located in the promoter region associated with the greatest relative increase in activation by Ets-1) were able to bind Ets-1 protein, regardless of whether they were expressed as recombinant protein in vitro or in PMA-stimulated living cells. The observation of higher molecular mass complexes using nuclear extracts of PMA-stimulated

EA.hy926 cells can be explained either by proteolysis, truncated forms of recombinant Ets-1 protein, or, much more likely, by binding of a coactivator protein to DNA-bound Ets-1, which has previously been reported for cAMP-responsive element binding-binding protein/p300 (Yang et al., 1998).

Strong activation of the ECE-1a promoter by mutation of EBS −638 is highly suggestive of binding of a strong transcriptional repressor that most probably is also a member of the ETS family. ETS proteins, such as SAP2, NERF-1 or ERF, have been identified as transcriptional repressors (Sharrocks et al., 1997). The factor responsible for repression of the ECE-1a promoter in human endothelial cells remains to be identified by further experimentation. Among ETS proteins other than Ets-1, NERF-2 was shown to be expressed in endothelial cells, where it regulates the gene encoding the receptor tyrosine kinase *tie*-2 (Dube et al., 1999). However, NERF2 has not been recognized as a transcriptional repressor. Mutation of EBS −638 also diminished PMA effects by 80%, which further underlines that PMA effects on ECE-1a expression are essentially mediated by Ets-1. We conclude that under stimulation conditions investigated in this work, either PMA stimulation or Ets-1 coexpression, de novo synthesized Ets-1 protein is likely to activate the ECE-1a promoter by two closely linked mechanisms, derepression by displacement of a putative repressor and *trans*-activation.

We found that the effects of PMA-induced PKC activation were suppressed using the specific MEK1/2 inhibitor PD98059. Importantly, PD98059 was excluded to inhibit PKC at the concentrations used in our experiments (Alessi et al., 1995). A functional link between PKC and the MEK1/2-p44/p42 pathway as observed in this study has been demonstrated in endothelial, but also other cell types. For example, induction of cyclooxygenase-2 mRNA expression in HUVEC by PMA was suppressed by PD98059 (Hirai et al., 1999). The results of our MEK1/2 inhibition experiments imply that ECE-1a mRNA expression may be physiologically induced by converging signaling pathways: 1) activation of PKC by diacylglycerol released from phospholipase C activation via G-protein-coupled receptors, 2) activation of the Raf-MEK1/2-p44/p42 by receptor tyrosine kinases, or 3) Ras-Raf interaction related to activation of G-protein-coupled receptors.

Knowledge about physiologic activators of endothelial ECE-1 expression is limited. Previously, induction of ECE-1 expression by vascular endothelial growth factor in cultured bovine aortic endothelial cells was reported, but ECE-1 isoforms were not explored in this work (Matsuura et al., 1997). A recent report demonstrated that angiotensin II and epinephrine were able to enhance ECE-1 protein expression in cultured HUVEC only in the presence of preeclamptic serum (Nishikawa et al., 2001). This suggests that stimulation of ECE-1 expression in vitro may be achieved only when a complex "cocktail" of biological stimuli is applied as found in pathophysiology in vivo.

Some pathophysiological situations associated with increased expression of ECE-1 in the vascular wall have been reported. In the rat carotid artery model of neointima formation, increased levels of ECE-1 mRNA were demonstrated in injured vessels at 12 and 24 h after injury (Wang et al., 1996). The kinetics of ECE-1 expression reported in that study is similar to our own observations in vitro and, therefore, leads us to hypothesize that in vivo ECE-1a expression may be up-regulated in endothelial cells located at the wound edge by the transcriptional mechanism proposed in this article. In the same in vivo model, maximum expression of ECE-1 mRNA was detected at day 5 after injury, and, at this time point, expression of ECE-1 protein was localized mainly in neointimal cells (Minamino et al., 1997). However, isoform-specific analysis of ECE-1 expression was not investigated. Based on the previously reported induction of Ets-1 expression by ET-1 in cultured VSMC (Hultgardh-Nilsson et al., 1996), we speculate that an auto-paracrine feedback mechanism may exist that links increased release of ET-1 (due to up-regulation of ECE-1 expression) to the induction of Ets-1 in VSMC and increased ECE-1a expression.

Augmented expression of ECE-1 in VSMC, but also in macrophages, was detected in experimental arteriosclerosis (Grantham et al., 1998) and in arteriosclerotic lesions of human coronary arteries (Minamino et al., 1997). Furthermore, reduction of ECE activity by the NEP inhibitor, candoxatril, was associated with decreased tissue ET-1 level and reduced atheroma formation in cholesterol-fed rabbits (Grantham et al., 2000). Regarding ECE-1 isoform expression in VSMC in vitro, expression of isoforms ECE-1b/c, but not ECE-1a, was reported under control conditions and also after stimulation with tumor necrosis factor-α and interferon-γ, respectively (Woods et al., 1999). The relevance of ECE-1 isoform expression in VSMC remains a matter of debate, because down-regulation of ECE-1c in bovine pulmonary artery smooth muscle cells did not result in a decrease of ET-1 release (Barker et al., 2001). Other studies, however, associated increased expression of ECE-1a and of ET-1 with morphological and functional alterations in diseased human peripheral arteries (Rossi et al., 1999). We therefore conclude that the definitive pathophysiological impact of ECE-1 depends on the specific cellular expression pattern of ECE-1 isoforms.

Regarding the regulation of ECE-1a by Ets-1 in endothelial cells in vivo, it is tempting to speculate that this mechanism may be activated during angiogenesis. Up-regulation of Ets-1 expression in endothelial cells was observed during embryogenesis, in endothelial cells of granulation tissue, and in capillary endothelial cells of tumor vessels (Wernert et al., 1992). Several reports support a role for the endothelin system in angiogenesis; recently, ET-1 was shown to augment the angiogenic potential of HUVEC in vitro and in vivo (Salani et al., 2000).

In summary, our work provides detailed insight into the transcriptional mechanisms that underlie PKC-dependent regulation of isoform-specific *ECE-1* gene expression in cultured human endothelial cells. Our findings provide the first evidence of a functional link between the endothelin system and the ETS family of transcription factors, which may contribute to initiation or progression of vascular disease in vivo.

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