Constitutive Phosphorylation of Human Endothelin-converting Enzyme-1 Isoforms*

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To investigate the phosphorylation of human endothelin-converting enzyme-1 (hECE-1) and identify potential residues involved, both in vivo and in vitro phosphorylation labeling assays of hECE-1 isoforms were performed in combination with site-directed mutagenesis and mass spectrometric analyses. Initial studies found that endogenous hECE-1 was constitutively phosphorylated in a primary endothelial cell line. The four known isoforms of hECE-1 expressed in this cell line (1a, 1b, 1c, and 1d) were then cloned by reverse transcription-PCR to determine which isoform(s) may be phosphorylated. The isoforms differ only in the first portion of their short amino-terminal cytoplasmic domains whereas their transmembrane domains and ectodomains of the proteins are identical. Isoforms 1b, 1c, and 1d but not 1a were constitutively phosphorylated in vivo when expressed in Chinese hamster ovary cells and casein kinase I readily phosphorylated the immunopurified isoforms in vitro. Site-directed mutagenesis established that two conserved serine residues, Ser18 and Ser30, (numbering based on isoform 1c) form at least one phosphorylation site in these three isoforms. Mutant forms of 1b, 1c, and 1d were constructed in which a single alanine was introduced at either serine residue and a double mutant for each isoform was constructed as well in which both serines were replaced with alanine. Phosphorylation of the single mutants was greatly reduced and was nearly abolished in the double mutants in both in vivo and in vitro labeling assays. Analysis by MALDI-MS of 32P-labeled proteolytic peptides derived from wild type 1c and the 1c mutants supported both Ser18 and Ser30 as phosphorylated residues. These data demonstrate the first finding that hECE-1 is constitutively phosphorylated within its cytoplasmic domain in an isoform-specific manner.

Endothelin-converting enzyme (ECE) is a zinc-binding metalloprotease responsible for the final proteolytic processing step in the biosynthesis of endothelins (ETs) (1), potent vasoconstrictive and mitogenic substances produced mainly by endothelial and smooth muscle cells. ECE may be involved in the synthesis and/or degradation of other peptide hormones as well (2, 3). Three endothelin peptides have been identified (ET-1, ET-2, and ET-3) (4). ETs are produced as large preproendothelin polypeptides, containing ~200 amino acids. Following removal of the signal peptides, the inactive intermediate big ET is formed consisting of 38–41 amino acids. ECE then cleaves big ET between Trp21 and Val22/Ile22 to generate the mature active 21-amino acid protein (1, 5). The importance of this cleavage site is demonstrated by the fact that the vasoconstricting activity of either ET-1-(1–20) amino acids or ET-1-(1–22) amino acids is three orders of magnitude weaker than ET-1-(1–21) amino acids (6). The enzyme therefore plays a key role in maintaining vascular tone by catalyzing the production of ET, making it an important target for the treatment of pathological conditions such as cardiovascular and renal diseases.

ECE belongs to the M13 subfamily of neutral endopeptidases. The mammalian subfamily consists of eight other members, including nephrilysin (NEP) (7), the erythrocyte cell-surface antigen Kell (KELL) (8), and a phosphate-regulating neutral endopeptidase (PEx) (9). These enzymes are type-II integral membrane proteins with a short amino-terminal cytoplasmic domain followed by a single transmembrane domain and a large carboxyl-terminal domain containing the active site. Two isoenzymes of ECE have been cloned and characterized, ECE-1 (1, 5, 10, 11) and ECE-2 (12). The overall amino acid sequence identity of ECE-1 to ECE-2 is 59% and they differ in substrate specificity, pH optimum, cellular localization, and tissue distribution.

Four isoforms of human ECE-1 (1a, 1b, 1c, and 1d) have been cloned. The four proteins are encoded by one gene and share a common carboxyl-terminal portion. Each is expressed from one of four distinct promoters that regulate expression of four unique amino termini (13–16). Although the ectodomain containing the active site is identical in each of the isoforms, the diverse amino-terminal sequences may be responsible for differences in tissue-specificity of expression as well as differences in subcellular localization. The different isoforms are localized to either the cell surface or to intracellular compartments, possibly the Golgi complex and Weibel-Palade bodies, regulated storage granules found in endothelial cells (1, 14, 16–22).

All four isoforms of hECE-1 contain several consensus recognition sequences for a variety of kinases. In addition, isoforms 1b, 1c, and 1d contain isoform-specific phosphorylation sequences in their unique amino termini for CK-I, CK-II, cAMP-dependent protein kinase (PKA), and protein kinase C (PKC). Given that phosphorylation plays a critical role for the proper function and/or localization of numerous proteins, we wished to investigate whether any or all of the hECE-1 isoforms are

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‡ The abbreviations used are: ECE-1, endothelin-converting enzyme-1; C12E10, polyoxyethylene-10-lauryl ether; CHO, Chinese hamster ovary; CK-I, casein kinase-I; CK-II, casein kinase-II; ET-1, endothelin-1; HUVECs, human umbilical vein endothelial cells; MALDI, matrix-assisted laser-desorption ionization; MS, mass spectrometry; NEP, nephrilysin; PAS, protein A-Sepharose; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; RT-PCR, reverse transcription-PCR; HPLC, high performance liquid chromatography.
Phosphorylation of hECE-1 Isoforms

In order to assess phosphorylation of hECE-1, we performed in vivo metabolic labeling with [32P]orthophosphate in human umbilical vein endothelial cells (HUVECs). We then cloned the isoforms 1b, 1c, and 1d by RT-PCR using total RNA isolated from HUVECs. Each isoform was expressed in CHO cells for in vivo phosphorylation studies, and the immunopurified proteins were used for in vitro kinase assays. Site-directed mutageneis of the phosphoamino acids were constructed to analyze phosphorylation of ECE-1.

**EXPERIMENTAL PROCEDURES**

Materials—Pefabloc, pepstatin, and leupeptin were purchased from Roche Molecular Biochemicals. [γ-32P]ATP and [32P]orthophosphate were purchased from PerkinElmer Life Sciences and Tran-32S-labeled ECE was purchased from ICN Biomedicals. Peptide N-glycosidase F (PNGase F), a protein phosphatase, CK-I, CK-II, and PKA were purchased from New England Biolabs. PKC was purchased from BioMol. Polyoxylethylene-10-lauryl ether (C12E10), sodium orthovanadate, fenvalerate, and Microcystin-LR were from Calbiochem. The FLAG peptide and anti-FLAG M2-agarose affinity gel were purchased from Sigma. Precast SDS-polyacrylamide gels were from Novex. All oligonucleotides were custom synthesized by Invitrogen and MWG Biotech.

Cell Culture—Pooled HUVECs were grown in EGM-2 growth medium supplied by the manufacturer (Clonetics). CHO-K1 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Stable cell lines expressing FLAG-ECE-1 isoforms were generated by transfecting CHO cells with the pZeoSV/FLAG-ECE-1a vector. ECE-1 isoforms were then subcloned using the TOPO PCR kit (Invitrogen). Plasmids with the correct insert were then subjected to a second round of PCR using the specific primers: hECE-1b, 5′-TTAATTATGATGTCGACGTACAAGCGG-3′ (Spe1 site) and FLAG-ECE-1a, 5′-GGACTAGT ATG GCT CGG GGC GTG TGG CCG-3′ (HindIII site); FLAG-ECE-1c, 5′-GCT CGG GGC GTG TGG CCG-3′ (HindIII site) and hECE-1c, 5′-TTAATTATGCGGGGCGTGTGGCCG-3′ (HindIII site). PCR was performed using Pfu Turbo (Stratagene) and MasterAmp PCR Optimization Kit with ammonium sulfate (Epitend). PCR fragments were gel-purified and subcloned using the TOPO PCR kit (Invitrogen). Plasmids with the correct inserts were then subjected to a second round of PCR using the above antisense primer and isoform-specific primers containing a unique EcoRI restriction site and tag sequence: FLAG-ECE-1b, 5′-GGACTAGT ATG GCT CAT AAG GAT GAC GAT GAC AAG GCT CGG GGC GTG TGG CCG-3′; FLAG-ECE-1c, 5′-GGACTAGT ATG GCT CAT AAG GAT GAC GAT GAC AAG GCT CGG GGC GTG TGG CCG-3′; FLAG-ECE-1d, 5′-GGACTAGT ATG GCT CAT AAG GAT GAC GAT GAC AAG GCT CGG GGC GTG TGG CCG-3′. PCR fragments were then gel-purified and subcloned into the TOPO vector. ECE-1 isoforms were then subcloned using SpeI and EcoRV sites into the pZeoSV/FLAG-ECE-1a vector. Plasmids were transformed into the E. coli strain TOP10 and grown overnight at 37 °C. The resulting plasmids were purified by restriction analysis and DNA sequencing, and subcloned into the pZeoSV/FLAG-ECE-1a vector. ECE-1 isoforms were then subcloned using SpeI and EcoRV into a wild type pZeoSV/FLAG-ECE-1 plasmid that had not been subjected to mutagenesis.

Metabolic Labeling, in Vivo Phosphorylation, and Immunoprecipitation of ECE-1—Metabolic labeling with Tran-[32S]methionine was performed as described previously (23). For in vivo phosphorylation, CHO/FLAG-ECE-1 cells were incubated for 1 h in phosphate-free Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% dialyzed fetal bovine serum, and then labeled with 0.2–0.8 mCi of [32P]orthophosphate for 5 h. Plates were washed with ice-cold phosphate-buffered saline, and lysed with radiolabeled immunoprecipitation assay (RIPA) buffer containing protease inhibitors (1 mM Pefabloc, 1 µg/ml pepstatin A, and 50 µg/ml leupeptin) as well as phosphatase inhibitors (10 µM sodium orthovanadate, 100 µM fenvalerate, 1 mM Microcystin-LR). Lysates were preincubated by incubating with protein A Staphylococcus aureus (PAS) beads (Sigma) for 1 h at 4 °C. For labeling in HUVECs, ECE-1 was immunoprecipitated using a monoclonal anti-ECE-1 antibody (ECE-6). Immunoprecipitation of FLAG-ECE-1 was carried out with an anti-FLAG M2-agarose affinity gel (Sigma). All samples were separated by 8% Tris-glycine SDS-PAGE. Gels were fixed in 30% methanol/7.5% acetic acid, dried, and exposed to Biomax MR or MS film (Kodak).

Immunoprecipitation of FLAG-ECE-1—FLAG-ECE-1 was eluted from the resin using 1 µg/ml FLAG peptide (Sigma). The eluted samples were concentrated and buffer exchanged using a Centricron YM-30 (Amicon/ Millipore) and aliquots used for in vitro kinase assays.

In Vivo Phosphorylation and Immunoprecipitation of ECE-1—FLAG-ECE-1 isoforms were treated with λ protein phosphatase following the manufacturer’s protocol (NEB). FLAG-ECE-1 was immunoprecipitated from the reaction with anti-FLAG M2-agarose affinity gel, eluted by competition with purified flag peptide, concentrated and buffer exchanged as described above. Purified FLAG-ECE-1 was incubated with CK-I, CK-II, PKC in the appropriate reaction buffers with [γ-32P]ATP for 30 min at 30 °C following the manufacturer’s recommendations (NEB and BioMol). The reactions were stopped with the addition of EDTA to a final concentration of 5 mM. The samples were buffer exchanged and [γ-32P]ATP was removed using a Microcon YM-10 spin column (Amicon/Millipore). Laemmli sample buffer was added, the samples were subjected to 8% Tris-glycine SDS-PAGE, and analyzed by autoradiography.

Phosphopeptide Mapping by MALDI-MS—[32P]-labeled FLAG-ECE-1 was immunoprecipitated from CHO cells as described above, separated by 8% Tris-glycine SDS-PAGE, and the 130-kDa band corresponding to phosphorylated ECE-1 was excised. After partial dehydination, the gel slice was incubated overnight at 37 °C with 10 µg of Promega sequence grade trypsin in 50 mM ammonium bicarbonate, pH 8.0. The soluble peptides in the digest were separated using an RP-HPLC system equipped with a BioResources Ultra Microcystin-18 BioUltra HPLC column. A 2 µg aliquot of the protein was then bound to a 1 x 15 mm ReliaSIL C18 column and eluted by a gradient of 5–65% (v/v) acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 50 µl/min in 23 or 55 min. The eluant was monitored with UV absorbance at 214 nm, and fractions were collected throughout the gradient at 1-min intervals. To analyze 32P incorporation, an aliquot of each fraction was either counted in a liquid scintillation counter (Amersham Biosciences) or spotted on a TLC plate and analyzed using a PhosphorImager (Molecular Dynamics). The radioactive fractions were then analyzed by MALDI-MS to characterize the phosphopeptides.

For MALDI analysis, a 0.5–µl aliquot of each labeled HPLC fraction was added to 0.5 µl of a saturated 1-cyano-4-hydroxycinnamic acid matrix solution containing a mixture of 0.1% trifluoroacetic acid and acetonitrile (1:1). The solution was allowed to dry before introduction into the mass spectrometer. Spectra were acquired on a Voyager-DE STR MALDI-TOF mass spectrometer (Perseptive Biosystems, Inc.), with a nitrogen laser (337 nm, 3-ns pulse). Each spectrum was produced in the linear mode by accumulating data from 250 to 1000 laser shots. Time-to-mass conversion was achieved by calibration using bradykinin (MH+ at m/z 1661.2 Da) and ACTH (18–39) (MH+ at m/z 2466.72 Da). The program prospector.ucsf.edu was used to calculate the masses of all possible phosphopeptides and phosphopeptides from ECE-1 and its mutants. Dephosphorylation of the HPLC fractions containing phosphopeptides was achieved by incubation of the peptide fraction with 2 units of alkaline phosphatase (NEB) in 50 mM NH4HCO3, pH 8.0, at 37 °C for 4 h. Desalting of the dephosphorylated samples was accomplished with C18 zip tip (Millipore Corp.).
RESULTS

Constitutive Phosphorylation of hECE-1 in HUVECs—The amino acid sequence for hECE-1 contains several potential kinase recognition sites. To investigate the possibility that these or other phosphorylation sites may be present in ECE-1, we first wanted to determine whether phosphorylation of ECE-1 occurs in a cell line expressing endogenous ECE-1. HUVECs, known to express message for all four ECE-1 isoforms, were labeled with [35S]methionine and immunoprecipitated using a monoclonal ECE-1 antibody, ECE-6. Immunoprecipitates were then analyzed by SDS-PAGE and autoradiography as described under “Experimental Procedures.”

Fig. 1A shows the autoradiograph of the labeled immunoprecipitates. ECE-1, metabolically labeled in HUVECs with [35S]methionine and immunoprecipitated, served as a marker for ECE-1, which has a molecular size of ~130 kDa when analyzed by SDS-PAGE (Fig. 1A, lane 1). Similarly, [32P]orthophosphate labeling of HUVECs and immunoprecipitation of ECE-1 resulted in a band of ~130 kDa (Fig. 1A, lane 3). The control immunoprecipitation was without antibody (Fig. 1A, lane 2). Although all three samples were prepared at the same time and run on the same gel, the samples in Fig. 1A are presented as two separate panels because a longer exposure was necessary to visualize the [35S]ECE-1 band whereas the [32P]-labeled ECE-1 was visible by autoradiography within 4 h. The identity of the bands as ECE-1 was confirmed by removing aliquots of the immunoprecipitates and immunoblottling using a polyclonal anti-ECE-1 antibody (Fig. 1B, lanes 1–3). These results indicated that ECE-1 might be constitutively phosphorylated in vivo. Phosphorylation of ECE-1 in HUVECs did not appear to be dependent upon PKA or PKC stimulation. Treatment of HUVECs with various concentrations of either forskolin or phorbol-12-myristate-13-acetate (PMA) following time courses did not appear to increase [32P]incorporation (data not shown). In addition, treatment with okadaic acid, which inhibits protein phosphatases 1 and 2A, did not significantly increase labeling indicating that the protein does not undergo rapid dephosphorylation in vivo. These results further suggested that hECE-1 might be constitutively phosphorylated.

The monoclonal antibody used for immunoprecipitation of ECE-1 recognizes the carboxyl terminus of hECE-1 and therefore does not differentiate between the four hECE-1 isoforms, 1a, 1b, 1c, and 1d. In order to distinguish which of the isoforms could be phosphorylated, we next cloned isoforms 1b, 1c, and 1d by RT-PCR. The three isoforms as well as isoform 1a were expressed in CHO cells for further characterization.

Cloning and Protein Expression of FLAG-ECE-1 Isoforms—The amino acid sequence alignment of the cytoplasmic amino termini of the four hECE-1 isoforms is shown in Fig. 2. The four isoforms are produced from the same gene using four distinct promoters (13–16). The proteins differ only in their immediate amino-terminal sequences (approximately the first 40 amino acids) whereas their transmembrane domains and ectodomains are identical. cDNAs for the hECE-1 isoforms 1b, 1c, and 1d were obtained using RT-PCR as described under “Experimental Procedures.” Briefly, total RNA from HUVECs was isolated and reverse transcribed using an ECE-1 antisense-specific primer. cDNAs were then subjected to PCR using isoform-specific primers to generate each of the isoforms 1b, 1c, and 1d. A FLAG epitope tag was placed at the amino terminus of each isoform by a second round of PCR. Constructs were subcloned into the mammalian expression vector pZeoSV and transfected into CHO cells. Two days following transfection, ECE-1 expression was analyzed by both metabolic labeling with [35S]methionine and immunoprecipitation of ECE-1, and by immunoblot analysis of cell lysates using a polyclonal antibody to ECE-1 (Fig. 3).

Cells were transiently transfected with the FLAG-ECE-1 constructs and labeled with [35S]methionine for 5 h. Cell lysates were prepared and FLAG-ECE-1 was immunoprecipitated using an anti-FLAG M2-agarose affinity gel. The immunoprecipitates were then analyzed by SDS-PAGE and autoradiography. CHO cells transfected with pZeoSV vector alone served as a negative control (Fig. 3, upper panel, lane 1). Each isoform produced two bands of ~130 kDa and 110 kDa, respectively (upper panel, lanes 2–5). Previous studies have demonstrated that the lower 110-kDa band represents an immature endoplasmic reticulum-associated form of ECE-1 and the 130-kDa protein a mature form of the enzyme (23). The identity of the 130-kDa band as ECE-1 was confirmed by immunoblot analysis using a polyclonal antibody to ECE-1 (lower panel, lanes 1–5). These constructs were used to make stable CHO cell lines (CHO/FLAG-ECE-1 cells) as described under “Experimental Procedures” for further labeling studies and
characterization of the isoforms. In addition, enzyme activity assays were performed on the FLAG-ECE-1 isoforms, as previously described (23), to confirm that the proteins were active.

In Vivo Phosphorylation of hECE-1 Isoforms—In order to determine which isoforms may be phosphorylated in vivo, CHO/FLAG-ECE-1 cells were labeled with \[^{32}P\]orthophosphate for 5 h and the isoforms immunoprecipitated using an anti-FLAG M2-agarose affinity gel. The immunocomplex was analyzed by SDS-PAGE and autoradiography as shown in Fig. 4 (upper panel). To monitor expression of the isoforms, aliquots of the immunoprecipitates were again analyzed by immunoblot using a polyclonal anti-ECE-1 antibody (Fig. 4, lower panel). FLAG-ECE-1a labeled with \[^{35}S\]methionine was included as a marker for ECE-1 on the gel (lane 1). CHO cells transfected with pZeoSV vector alone served as a negative control (lane 2).

Three of the isoforms appeared to be labeled in the presence of \[^{32}P\], although to varying degrees (lanes 3–6). Isoform 1c consistently appeared to have the strongest labeling, followed by 1d and to a lesser degree 1b. It should be noted that upon longer exposure times of the autoradiograph, isoform 1a also appeared to be very weakly labeled by \[^{32}P\]. The presence of okadaic acid in the labeling media did not appear to greatly enhance the labeling of the isoforms and did not change the pattern of labeling (data not shown). These data indicated that ECE-1 isoforms may be constitutively phosphorylated in vivo and the degree of phosphorylation may be isoform-specific.

Identification of Kinases Involved in Phosphorylation of ECE-1—To identify a kinase involved in ECE-1 phosphorylation, we performed in vitro phosphorylation assays using immunopurified FLAG-ECE-1 as described under “Experimental Procedures.” Briefly, FLAG-ECE-1 isoforms expressed in CHO cells were immunopurified using an anti-FLAG M2-agarose affinity gel, eluted from the resin with purified FLAG peptide, and treated with a protein phosphatase. The dephosphorylated proteins were immunopurified from the reaction using an anti-FLAG affinity gel and eluted with purified FLAG peptide. The
Identification of the Phosphorylation Sites—To identify the residue(s) being phosphorylated we performed site-directed mutagenesis. Because the degree of phosphorylation appeared to be isomeric-specific, we first focused on the differences between the isoforms that reside in their cytoplasmic tails (refer to Fig. 2B for amino acid sequences). Sequence alignment of the amino termini of the isoforms and a phosphorylation prediction program revealed one unique CK-I recognition site for isoform 1b (SALLSALG) and another predicted CK-I site common to all four isoforms (SPRSGQR). In order to identify if these serine residues or other residues were involved in phosphorylation, mutations of the cytoplasmic tail of isoform 1c (the isoform most heavily phosphorylated) were constructed in which a single alanine was introduced at either one of the five serines, three threonines, or one tyrosine found in the amino terminus. In addition, a double mutant was constructed in which the two serines identified in the sequence above (SPRSGQR), corresponding to Ser35 and Ser38 in 1c, were both mutated to alanine, S35/38A. Mutation of the residues to alanine was carried out as described under “Experimental Procedures,” the constructs expressed in CHO cells, radiolabeled with [32P]orthophosphate, the flag-tagged proteins immunoprecipitated and analyzed by autoradiography as described above.

Phosphorylation of the mutants appeared to be similar to wild type (WT) levels however, two serine residues appeared to be necessary for in vitro phosphorylation of 1c, Ser18 and Ser20 (Fig. 6A, upper panel). WT-1c phosphorylation is shown in lane 1. The single mutant S18A appeared to be slightly less phosphorylated than WT-1c, and labeling of the S20A mutant was considerably lower than WT-1c (lanes 2 and 3). The double mutant S18A/S20A nearly abolished phosphorylation (lane 4). Protein expression was again analyzed by immunoblotting aliquots of the immunoprecipitates (lower panel). These results suggested that Ser18 and Ser20 may be phosphorylation sites for isoform 1c.

In vitro kinase assays also confirmed that these two serine residues form a recognition site for CK-I (Fig. 6B). The mutant proteins were expressed in CHO cells, immunopurified using an anti-FLAG M-2 agarose affinity gel and treated with λ protein phosphatase as described earlier. The dephosphorylated proteins were incubated with CK-I and [γ-32P]ATP, subjected to 8% Tris-glycine SDS-PAGE and analyzed by autoradiography. WT-1c phosphorylation is shown in Fig. 6B, lane 1. Phosphorylation of the single mutants S18A and S20A as well as the double mutant S18A/S20A was considerably lower than...
Fig. 6. Identification of phosphorylation sites in ECE-1c, -1b, and -1d by site-directed mutagenesis. A, CHO cells expressing FLAG-WT-1c or the FLAG-ECE-1 mutants were labeled with 0.5–1.0 mCi of [32P]orthophosphate, and cells were processed as described under “Experimental Procedures.” FLAG-ECE-1 was immunoprecipitated from lysates, subjected to 8% Tris-glycine SDS-PAGE and exposed to Biomax MS film (upper panel). Labeling of cells expressing WT-1c (lane 1) is shown. Phosphorylation of the single mutants S18A and S20A, and the double mutant S18A/S20A was analyzed (lanes 2–4). To examine the immunoprecipitation of the ECE-1 mutants, aliquots from samples were subjected to 8% Tris-glycine SDS-PAGE, followed by immunoblot analysis using a polyclonal anti-ECE-1 antibody (lower panel). B, in vitro assays were also performed on FLAG-WT-1c and the mutants to identify these residues as a phosphorylation site for CK-1. Phosphatase-treated immunopurified FLAG-ECE-1 proteins were incubated with CK-1 in the presence of γ-[32P]ATP as described under “Experimental Procedures.” Labeling of WT-1c is shown in lane 1 and labeling of the 1c mutants in lanes 2–4. C, the corresponding serine residues in isoforms 1b and 1d were also mutated. Phosphorylation of WT-1b, WT-1d, and the mutants for each of the isoforms, S34A, S36A, S34A/S36A, S31A, S33A, and S31A/S33A was assessed by in vivo [32P]orthophosphate labeling as described in panel A (upper panel, lanes 1–8). Aliquots of the immunoprecipitations were used for immunoblot analysis (lower panel, lanes 1–8).

Phosphorylation of WT-1c (lanes 2–4). These results demonstrated that Ser18 and Ser20 make up at least one phosphorylation site in ECE-1c and that the residues are phosphorylated by CK-1 in vitro.

We next wanted to determine whether the corresponding serine residues in isoforms 1b and 1d were phosphorylated. The conserved serine residues in 1b and 1d were mutated to alanine and phosphorylation of the mutants assessed by in vivo labeling in CHO cells, immunoprecipitation, and autoradiography. For isoform 1b the single mutations S34A and S36A, and the double mutant S34A/S36A were constructed, and for isoform 1d, the mutants S31A, S33A, and S31A/S33A were made (refer to Fig. 2B for sequences and numbering). As seen with isoform 1c in Fig. 6A, the single mutants showed decreased labeling compared with WT-1b and WT-1d, and the double mutations nearly abolished phosphorylation of ECE-1b and 1d (Fig. 6C, upper panel, lanes 1–8). The lower panel shows the immunoblot of the immunoprecipitated samples. These results demonstrated that the two conserved serine residues in the cytoplasmic tail of isoforms 1b, 1c, and 1d create at least one phosphorylation site in ECE-1.

Identification of the Phosphorylation Sites Using Mass Spectrometry—To further characterize the phosphorylation sites, MALDI-MS was used to identify potential phosphorylation sites in WT-ECE-1c and the ECE-1c mutants S18A, S20A, and S18A/S20A. Briefly, [32P]labeled FLAG-ECE-1 was immunoprecipitated from CHO cells, separated by 8% Tris-glycine SDS-PAGE, and the 130-kDa band corresponding to phosphorylated ECE-1 was excised. The protein was digested with trypsin and the digest separated using RP-HPLC. Radioactive HPLC fractions were analyzed by MALDI-MS. The masses of the 32P-labeled tryptic peptides from the site-directed mutants S18A and S20A were identified and confirmed by MALDI-MS. Each MALDI spectrum of the radioactive fractions was examined to identify if a difference of ±80 daltons (or multiples of 80 Da) was observed between two peaks, which would correspond to the difference between the phosphorylated and dephosphorylated peptide.

Phosphorylation of Ser18 and Ser20 was supported by MALDI-MS on the radioactive HPLC fractions of a tryptic digest of WT-ECE-1c (Fig. 7A). This spectrum shows a peak at m/z 3448.21, which corresponds to the MH+ ion of the tryptic fragment ECE-1c-(8–37) with two phosphorylated residues (calculated MH+ at m/z 3448.46–3450.4). Treatment of this HPLC fraction with alkaline phosphatase caused the peak at m/z 3448.21 to disappear (data not shown), consistent with the assignment of the phosphopeptide with phosphates on both Ser18 and Ser20. Although other potential phosphoresidues exist in this fragment (Thr25, Tyr25, and Ser35), earlier site-directed mutagenesis results indicated that these residues are not phosphorylated, suggesting that Ser18 and Ser20 are most likely the phosphoresidues. The MS data are also consistent with the mutagenesis data that two phosphorylation sites are occupied. Further support for this conclusion was obtained by MALDI-MS analysis of mutant protein digests.

The masses of the [32P]-labeled tryptic peptides from the site-directed mutants S18A and S20A were identified and confirmed by MALDI-MS. The peak in the spectrum of one labeled HPLC fraction from the digest of S20A (Fig. 7B) at m/z 3354.08 Da represents the mass of the Ser20 mutant changed to Ala20 with one phosphate remaining in the fragment, possibly on Ser18. Treatment of this HPLC fraction with alkaline phospho-
tase caused the peak at 3354.08 Da to disappear, indicating that this mass is due to the presence of the Ser18 phosphorylation. These data showing a peptide from the single mutant still having one remaining phosphoresidue are consistent with the earlier mutagenesis results and the assignment of the phosphoresidues.

Coincidentally, MALDI-MS analysis of one labeled HPLC fraction for the digest of the double mutant S18A/S20A revealed a mass which corresponds to the mass of the ECE-1c peptide 8–37 with two phosphorylation sites within the protein sequence. Other peaks in the spectrum correlate to tryptic peptides coeluting with the phosphorylated peptide in the fractionation of the tryptic digest of ECE-1c protein. B, MALDI-MS analysis of S20A mutant. The peak present in the MALDI spectrum at a mass of 3354.08 Da represents the peptide 8–37 of the single mutant S20A with one phosphorylation site within the protein sequence. Other peaks in the spectrum correlate to various tryptic peptides with the same retention as the single phosphorylated S20A peptide which coelute during the RP-HPLC fractionation of the tryptic digest of the S20A protein.

Effects of FLAG Epitope Tag on Phosphorylation of ECE-1—CK-I requires an acidic sequence motif for substrate recognition (reviewed in Ref. 30). To determine whether the presence of the acidic FLAG epitope tag (DYKDDDDK) at the amino terminus affected phosphorylation of the ECE-1 isoforms, we expressed constructs of the ECE-1 isoforms without a FLAG tag in CHO cells and labeled the cells with $^{32}$P[orthophosphate in vivo, immunoprecipitated, and separated on 8% Tris-glycine SDS-PAGE. The ECE-1 bands were excised, digested with trypsin, purified by RP-HPLC, and the $^{32}$P-containing HPLC fractions were then analyzed by MALDI-MS. A, MALDI-MS analysis of WT-ECE-1c fragments. The peak present in the MALDI spectrum at m/z 3448 corresponds to the mass of the ECE-1c peptide 8–37 with two phosphorylation sites within the protein sequence. Other peaks in the spectrum correlate to tryptic peptides coeluting with the phosphorylated peptide in the fractionation of the tryptic digest of ECE-1c protein. B, MALDI-MS analysis of WT-ECE-1c fragments. The peak present in the MALDI spectrum at m/z 3448 corresponds to the mass of the ECE-1c peptide 8–37 with two phosphorylation sites within the protein sequence. Other peaks in the spectrum correlate to tryptic peptides coeluting with the phosphorylated peptide in the fractionation of the tryptic digest of ECE-1c protein. B, MALDI-MS analysis of S20A mutant. The peak present in the MALDI spectrum at a mass of 3354.08 Da represents the peptide 8–37 of the single mutant S20A with one phosphorylation site within the protein sequence. Other peaks in the spectrum correlate to various tryptic peptides with the same retention as the single phosphorylated S20A peptide which coelute during the RP-HPLC fractionation of the tryptic digest of the S20A protein.

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Fig. 7. MALDI-MS analysis of a radioactive HPLC fraction from the tryptic digest of in vivo labeled ECE-1c. FLAG-ECE-1c proteins (wild type and the mutants) were labeled with $^{32}$P[orthophosphate in vivo, immunoprecipitated, and separated on 8% Tris-glycine SDS-PAGE. The ECE-1 bands were excised, digested with trypsin, purified by RP-HPLC, and the $^{32}$P-containing HPLC fractions were then analyzed by MALDI-MS.
Orthophosphate, and ECE-1 was immunoprecipitated using a monoclonal anti-ECE-1 antibody and analyzed by autoradiography and immunoblot (upper and lower panels, respectively).

and 1d were phosphorylated in CHO cells. The degree of phosphorylation between the isoforms however, appeared to be more uniform than the $^{32}$P labeling seen with the FLAG-tagged proteins (Fig. 8, lanes 2–5). Although addition of the FLAG epitope tag seemed to affect the degree of phosphorylation between the isoforms probably due to the acidic nature of the tag, the three isoforms without the tag were nevertheless phosphorylated, and a phosphorylated form of ECE-1 was found in HUVECs (Fig. 1). It could be that addition of the tag is making more accessible a site that is normally phosphorylated at a relatively low level. Certainly phosphorylation of the endogenous untagged form(s) of the enzyme in Fig. 1 seems clear.

**DISCUSSION**

The results described in this study demonstrate the first finding that hECE-1 is phosphorylated. Isoforms 1c, 1d, and 1b, and to a lesser degree 1a, appear to be constitutively phosphorylated in *vitro*. Phosphate incorporation was not increased by PKC or PKA stimulation. Based upon *in vitro* analysis, the kinase involved may be CK-I or a CK-I-like kinase. We determined a major phosphorylation site at Ser$^{18}$ and Ser$^{20}$ in isoforms 1c as well as the corresponding serine residues in isoforms 1b and 1d using site-directed mutagenesis. These data were also supported by MALDI-MS analysis on isoform 1c. The two serine residues are conserved in the cytosolic tails of isoforms 1c, 1b, and 1d, but not in isoform 1a, the most divergent isoform. Weak $^{32}$P labeling of 1a however, suggests that other phosphorylation sites may exist for the isoforms. Because the protein samples analyzed by MALDI-MS were recovered from small scale *in vitro* labeling experiments, the mass spectral data contained relatively weak signals from the HPLC fractions of the proteolytic digests. The lower intensity of these phosphopeptides (and their dephosphorylation products) in the presence of other peptides coeluting in the labeled HPLC fractions further exacerbated this problem and precluded more definitive localization studies using techniques such as post-source decay analysis.

ET-1 secretion is dependent upon numerous factors including hypoxia, epinephrine, angiotensin II, oncostatin, tumor necrosis factor-α, interleukin-1β, interferon-γ, and transforming growth factor-β (for review see Ref. 24). Each of these factors involves complex signaling pathways that often include tyrosine protein kinases or serine/threonine protein kinases. In addition, studies have demonstrated that mRNA levels and protein expression of ECE-1a may be up-regulated in response to PKC (25) but no previous studies have demonstrated that ECE-1 protein is directly modified by any kinases.

ECE-1 isoforms contain a number of phosphorylation consensus sequences indicating that the enzyme may be phosphorylated. NEP was reported to be phosphorylated by CK II *in vitro* and appeared to be phosphorylated *in vivo* on a serine or threonine (26). Although it was suggested using a soluble form of NEP that the phosphorylated residue(s) reside in the cytoplasmic tail, the phosphorylated amino acid(s) were not identified in the study. There have also been recent reports of other metalloproteases being modified by phosphorylation (27, 28), but none in the NEP/ECE family. Our results clearly demonstrate constitutive phosphorylation of ECE-1.

The subcellular localization of the four isoforms and their respective sites of activity remain unclear. Differences in localization of the isoforms may affect phosphorylation of the proteins. It is possible that phosphorylation may regulate activity, either directly or indirectly, in different parts of the cell affecting substrate specificity, as the pH optimum for ECE-1 activity is substrate-dependent. Small peptide hormones such as bradykinin and substance P have an acidic pH optima of 5.6–5.8, whereas big ET-1 has a neutral pH optimum, indicating that different substrates may be cleaved in different intracellular compartments (29). Isoforms localized to different subcellular areas may have different functions and phosphorylation of ECE-1 may be one way of regulating activity of the enzyme, directly or indirectly. A better understanding of the pathways involved and the processing of the various substrates is needed.

It is also important to note that many functions of ET and ECE depend upon cell types. Differences in mRNA levels of the four ECE-1 isoforms vary between cell types. For example, 1a is highly expressed in HUVECs but undetectable in HSMC (16). The different isoform-specific promoters are believed to relate to tissue-specific differences or to developmental or pathological differences. Phosphorylation of ECE-1 isoforms may also be dependent upon cell-type or physiological conditions. Results from this study indicate that ECE-1 is constitutively phosphorylated in HUVECs, which contain message of all four isoforms. It will be of interest to determine which isoform(s) this is as well as to determine phosphorylation patterns of ECE-1 endogenously expressed in other tissues and the conditions under which the proteins may be phosphorylated.

The results in this study also demonstrate that CK-I can phosphorylate ECE-1 *in vitro*. The initial consensus phosphorylation sequence for CK-I, S/T(P)X$^1$S/T(Y), was determined where S/T(P) is any phosphorylated serine or threonine upstream from the target amino acid (S/TY), and X is any amino acid. The phosphorylated S/T provides a favorable charge immediately amino-terminal to the target serine or threonine residue. Additional studies have revealed optimal sequences for non-phosphate-directed phosphorylation by CK-I isoforms (30). A cluster of more than three acidic residues upstream from position n-2 was required for optimal phosphorylation efficiency. For instance the tetrapeptide DDDD between n-6 and n-3 from the target serine served as a potent substrate. The phosphorylated serine residues in ECE-1b, 1c, and 1d all contain an extremely acidic region immediately amino-terminal to the phosphorylation sites identified in this study; DEEDLVDSLSE, agreeing with a predicted consensus sequence for CK-I. The sequence DEEDLVDSLSE found in hECE-1 is well conserved between several different mammalian species including bovine, guinea pig, and rat. A similar sequence consisting of serines flanked by acidic residues is found in the chicken ECE isoform (DDEDPLSISISED). The sequence is not found in mouse, hECE-1a, hECE-2, or hNEP. Because this region appears to be conserved in several mammalian species, phosphorylation of these serines may be a common event.
We have demonstrated that hECE-1 is constitutively phosphorylated in HUVECs and that isoforms 1b, 1c, and 1d are phosphorylated both in vivo and in vitro on two conserved serine residues found in their cytoplasmic tails. We have also provided evidence that CK-I phosphorylates the proteins in vitro. This is the first study showing phosphorylation of ECE-1 and identifies the residues involved. It will be interesting to determine which endogenous isoforms and residues are phosphorylated in other cell lines using isoform-specific antibodies. Further work will be required to define more completely the role of ECE-1 phosphorylation and possibly identify other phospho-residues in ECE-1.

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