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Protein Kinase C recognition sites in the cytoplasmic domain of Endothelin Converting Enzyme-1c

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ABSTRACT

Endothelin Converting Enzyme-1 (ECE-1) is essential for the production of the potent vasoconstrictor Endothelin-1 (ET-1). The activation of Protein Kinase C (PKC) by phorbol 12-myristate 13-acetate (PMA) can increase ECE-1 phosphorylation, which in turn promotes ECE-1c trafficking to the cell surface where ET-1 production occurs. This study has identified the specific residues in the N-terminal cytoplasmic tail of ECE-1c isoform that are phosphorylated upon the activation of PKC. Levels of phosphorylation are expressed as a % phosphorylation in untreated CHO-K1 cells. We transfected CHO-K1 cells with wild type and mutant forms of ECE-1c (Ala^4-ECE-1c, Ala^{35}ECE-1c and $Ala^{4/35}$ECE-1c) to confirm the involvement of Thr^4 and Ser^{35} residues in PMA induced phosphorylation of ECE-1c. Phosphorylation of wild type ECE-1c increased in response to PMA treatment (150 ± 13%, unpaired *t*-test, $P < 0.05$, significantly different compared to untreated control). The two single mutants and one combined mutant significantly reduced the PMA induced phosphorylation (103–117 ± 6–13%; unpaired *t*-test; $n = 8$; $P < 0.05$ significantly different compared to untreated control). The mutations had no effect on the basal ECE-1c phosphorylation. In addition, they had no effect on the catalytic activity as evidenced by the similar rate of substrate cleavage compared to wild type. This study is the first to confirm the residues phosphorylated upon the activation of PKC by PMA. The results complete a gap in our understanding of the mechanism(s) behind PKC induced trafficking of ECE-1.

1. Introduction

Endothelin-1 (ET-1) is the one of the most potent vasoconstrictors known thus far [1]. It is produced by the cleavage of its precursor Big Endothelin-1 (BigET-1) which is a 38 amino acid peptide with no biological activity [2]. BigET is cleaved between Trp21 and Val22 by the highly specific metalloprotease known as Endothelin Converting Enzyme-1 (ECE-1) to produce the 21 amino acid bioactive peptide ET-1 [2]. Therefore the expression and localisation of ECE-1 is the rate limiting step in the production of ET-1. ECE-1 (and therefore ET-1) has been implicated in the pathogenesis of a range of other diseases including cancer [3], cardiovascular [4–6] and Alzheimer's disease [7–9].

ECE-1 is a type II integral membrane bound protein with a large extracellular C-terminal domain, a short N-terminal domain and a single transmembrane region. The catalytic activity is confined to the C-terminal domain. Four isoforms (ECE-1a, b, c and d) of ECE-1 have been cloned and all are encoded by a single gene but under the control of different promoters [10,11]. The specific localisation of ECE-1 can affect its access to the BigET-1 substrate and hence ET-1 production. The only differences amongst the isoforms exists in the intracellular N-terminal region and these differences are attributed to the differential sub cellular trafficking of the ECE-1 isoforms [12]. Therefore phosphorylation can indirectly regulate ECE-1 activity in terms of ET-1 production [13,14]. The sequence of ECE-1 contains several sites for possible post-translational modifications including as many as 10 glycosylation sites [15], and palmitoylation [16] at a conserved cysteine residue close to the transmembrane domain. Phosphorylation of ECE-1 is thus far the most widely studied post translational modification [12,14,17].

Phosphorylation of ECE-1 can be described as either constitutive or stimulated. Previous work has shown that ECE-1 is constitutively phosphorylated at residues Ser^{18} and Ser^{20} (ECE-1c numbering) by Protein Kinase C (PKC) [17]. These residues are absent in ECE-1a and as such this isoform is therefore not constitutively phosphorylated [17]. The stimulated phosphorylation of ECE-1 is mediated by PKC as shown in previous studies conducted

Abbreviations: PMA, phorbol 12-myristate 13-acetate; CHO cells, Chinese Hamster Ovary cells; ECE-1, Endothelin Converting Enzyme-1; BigET-1, Big Endothelin-1; ATCC, American Type Culture Collection.

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by us [18,19] and others [7,20,21] where phosphorylation of ECE-1 is increased following the activation of PKC by phorbhol esters such as phorbol 12-myristate 13-acetate (PMA). This results in the trafficking of ECE-1 to the cell surface where the catalytically active ectodomain can be shed. [22]. Despite the confirmation of the sites of constitutive phosphorylation [17], the sites of PKC mediated (stimulated) phosphorylation are yet to be confirmed. Cytoplasmic tails of ECE-1 isoforms are known to contain potential recognition sites for a number of kinases [14,19]. Although not constitutively phosphorylated, we have previously identified Tyr^4 and Ser^{35} (ECE-1c numbering) as potential sites for recognition by PKC.

The stimulated phosphorylation of ECE-1 is likely to be the mechanism by which ECE-1 expression and localisation is regulated by physiological/pathophysiological factors. This is supported by studies which show that a high glucose environment can increase the expression of the ECE-1c isoform, a process which is sensitive to inhibitors of PKC [20]. Furthermore, high glucose mediated PKC activation results in the trafficking of ECE-1c to the cell surface [20]. Since ECE-1c is considered as a dominant isoform expressed in endothelial cells and hence is a principal regulator of blood pressure [19]. We have used CHO-K1 cells transfected with wild type and mutated forms of ECE-1c, to confirm the potential sites of recognition by PKC in the N-terminal domain of ECE-1c.

2. Material and methods

Ripa buffer in ddH_2O (50 mM TrisHCl, 100 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1% SDS, 0.5% deoxycholic acid sodium salt, 1% Triton X-100, 1 μg/ml aprotinin, 5 μg/ml leupeptin, 0.7 μg/ml pepstatin (SIGMA)).

Wash buffer 1 in ddH_2O (50 mM Tris–HCl, NaCl, 1% TritonX-100, 0.5% deoxycholic acid sodium salt, 1 μM PMSF, 1 μg/ml aprotinin, 5 μg/ml leupeptin, 0.7 μg/ml pepstatin (SIGMA)).

Wash buffer 2 in ddH_2O (50 mM Tris–HCl, 0.5 M NaCl, 0.1% TritonX-100, 0.05% deoxycholic acid sodium salt);

Wash buffer 3 in ddH_2O (50 mM Tris–HCl, 0.1% TritonX-100, 0.05% deoxycholic acid sodium salt).

2.1. Cell culture

CHO-K1, CHO-P, Cos-7, SWISS 3T3 and HEK 293 cells were obtained from American Type Culture Collection (ATCC). EA.hy926 (Endothelial cell line) were a generous gift from Dr. Edgell (Pathology Department, University of North Carolina, USA). The cells were maintained in media containing 50% α Mem, 40% foetal Calf Serum and 10% DMSO.

2.2. Transfections

For each well of a 12-well plate, 0.6 μg of plasmid DNA was diluted with 60 μl of OptiMem, whilst 4.8 μl of Lipofectamine was diluted with 60 μl of OptiMem. Both diluted solutions were mixed together and allowed to stand at room temperature for 20 min. After incubation, 480 μl OptiMem was added to DNA/Lipofectamine mixture, which was added to each well after washing with 2 ml of OptiMem. After 4–5 h incubation, cells were grown in complete media for a further 24–36 h.

2.3. Generation of a stable CHO-K1 cell line expressing ECE-1c

Cells were seeded into culture plates and transfected when 70% confluent. Transfection was conducted using lipofectamine transfection reagent and 1 μg of DNA (pcDNA3) encoding the ECE-1c sequence and previously linearised using the *Pvu* II restriction site. The next day, cells were trypsinized and split 10^4 fold into fresh culture plates containing the selective agent G_{418} (1 mg/ml, Gibco, USA). Media was changed twice a week until colonies of 100–200 cells were visible. Cells from these colonies were seeded onto 6 well plates and maintained in G_{418} containing α Mem for a further two weeks.

2.4. Generation of mutants

Single and double mutants of potential PKC phosphorylation sites were generated by substituting Thr^4 and/or Ser^{35} with alanine. The various mutations were introduced into the wild type ECE-1c expression vector using PCR-based site-directed mutagenesis (QuickChange, Stratagene). Oligonucleotides used for mutagenesis were (5′–3′):

- sense 1, GCCACCATGATGTCGGMCTACAAGCGGGCCACG;
- antisense 1, CGTGGCCC-GCTTGTAGKCCGACATCATGGTGGC;
- sense 2, CAGGTGAACTTCCAC-GMCCCGCGGAGTGGCCAGAGG;
- antisense 2, CCTCTGGCCACTCCGCG-GGKCGTGGAAGTTCACCTG.

Both set of primers contained wobble nucleotides (M = A or C, K = T or G) in order to produce either Ala or Asp. To help for screening of positive clones, the first site removed a *Sal*I site whereas a silent *Sac*II was generated by the second set of primers. The PCR product was *Dpn*I digested to remove parental DNA for 1 h at 37 °C. The nicked vector DNA incorporating the mutations was then transformed into DH5α *Escherichia coli*. Plasmid-bearing colonies (grown on Luria broth ampicillin plates) were screened for the relevant removed cut site or silent restriction site. Positive clones for each mutant were confirmed by sequence analysis (Micromon, Monash University, Australia).

CHO-K1 cells were then transfected and protein amounts quantified by Western analysis before the phosphorylation analysis of ECE-1c and its PKC site knockouts.

2.5. Phosphorylation studies

When transfected CHOK-1 cells were 70% confluent they were serum starved for 16 h. Following incubation with [^{32}P] Pi (80 μCi/well) in phosphate free medium (400 μl/well) for 2 h, cells were stimulated by PMA (2 μM) for 10 min at 37 °C. After stimulation cells were washed on ice twice with cold HBSS (1 ml/well). Membranes were solubilised with 0.3 ml/well of Ripa buffer. The lysates were harvested, centrifuged (14000 g for 30 min) and the supernatant pre-cleared by the addition of Protein A-agarose (10 μl) and BSA (6%, 10 μl) at 4 °C for at least 1 h. Pre-cleared lysates were then incubated overnight (4 °C) with 20 μl of Protein A-agarose beads and 2 μl of serum containing ECE-1 antibodies. The beads were washed, twice in each of wash buffers 1 & 2 twice followed by once in wash buffer 3. The immuno-precipitates were finally resuspended in 55 μl of SDS sample buffer, heated at 100 °C for 5 min and resolved by 8% SDS–PAGE. Gels were fixed, dried and exposed overnight against a Fuji-type BAS-IIIs PhosphorImaging plate. The plates were subsequently read in a FUJIX Bio-imaging Analyser BAS 1000 and the data analysed using MacBAS version 1.0.

2.6. ECE-1 activity assays

ECE-1 activity was measured based on the ability of the CHO-K1 cell lysates to cleave $BigET_{18-34}$ (DIIWVNTPEHVVPYGLG, Auspep, Vic, Australia) a truncated version of the natural substrate. $BigET_{18-34}$ (5 μg; dissolved in 10% DMSO and 90% TBS) was incubated in the presence of cell lysate. The reaction was stopped at time = 0 and 3 h by mixing an aliquot of the reaction mixture with