Applicability of green fluorescence protein in the study of endothelin converting enzyme-1c trafficking

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Abstract: Endothelin-1 (ET-1) is one of the most potent peptide vasoconstrictors known. It is produced upon the cleavage of its precursor big endothelin-1 by endothelin converting enzyme-1 (ECE-1). Production of ET-1 is thought to be dependent upon the expression of ECE-1 at the cell surface. Therefore, mechanisms inducing the trafficking of ECE-1 to the cell surface have been the focus of recent research. This research has identified phosphorylation of the cytoplasmic region of ECE-1 as a main cellular signal inducing its trafficking to the cell surface. Previous studies have used green fluorescent protein (GFP) tagged ECE-1 to monitor phosphorylation induced trafficking of ECE-1 to the cell surface. However, it has been speculated that the addition of the GFP tag can itself alter enzyme activity and phosphorylation of ECE-1, and hence the suitability of GFP or any other protein tag in studying ECE-1 distribution and trafficking. ECE-1c is the most widely expressed isoform in endothelial cells. We therefore expressed ECE-1c with a GFP tag either at the N or C-terminus of ECE-1c. Catalytic activity and effect on protein kinase C (PKC) induced phosphorylation was compared between the two chimeras and wild-type ECE-1c. Our results indicate that positioning of the GFP tag on the C-terminus abrogates activity without effecting PKC-induced phosphorylation. However, GFP tag on the N-terminus has the opposite effect. Results of this study shed light on the applicability of GFP or perhaps other protein tags in studying ECE-1c distribution and trafficking.

Keywords: endothelin-1; endothelin converting enzyme-1; green fluorescent protein; enzyme trafficking; protein kinase C; phosphorylation

Introduction

Endothelin converting enzyme-1 (ECE-1) is essential for the production of the potent vasoconstrictor hormone Endothelin-1 (ET-1). The enzyme is endogenously expressed on the surface of endothelial cells

where it cleaves Big Endothelin (BigET) between Trp21 and Val22 to produce 21 amino acid bioactive ET-1.¹ Therefore, the expression and localization of ECE-1 is thought to be the rate limiting factor in the production of ET-1.²

ECE-1 is a type II membrane bound metalloprotease consisting of a large extracellular C-terminus with catalytic activity, single transmembrane domain and a short N-terminal cytoplasmic domain.^{3,4} Four isoforms of ECE-1 (1a, 1b, 1c, and 1d) have been cloned and identified thus far. These isoforms are under the control of a single gene but different promoters.^{3,4} The only differences between the congeners are in the N-terminal cytoplasmic region, and these differences are attributed to influencing the different localization of each isoform.⁵ ECE-1c is

Abbreviations: ATCC, American type culture collection; BigET-1, big endothelin-1; ECE-1, endothelin converting enzyme-1; ET, endothelin; GFP, green fluorescent protein; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TBE, Tris-Borate-EDTA Buffer; TBS, tris buffered saline; TFA, trifluoro acetic acid.

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the predominant isoform expressed in endothelial cells and is therefore considered to be the principle regulator of blood pressure.⁶ The cytoplasmic N-terminus of ECE-1 is constitutively phosphorylated at Ser18 and Ser20.⁷ ECE-1a isoform lacks these residues and hence is not constitutively phosphorylated.⁷ The activation of protein kinase C (PKC) by phorbol esters such as phorbol 12-myristate 13-acetate (PMA), results in the phosphorylation of Tyr4 and Ser35 residues within the N-terminus of ECE-1.⁸ This has been widely accepted as a mechanism which induces the trafficking of ECE-1 to the cell surface.^{2,8-10} Disease stimuli such as high glucose have been proven to increase the trafficking of ECE-1c to the cell surface via this mechanism.⁹ Increased cell surface expression of ECE-1 has been implicated in the pathogenesis of cardiovascular diseases¹¹⁻¹³ and in female malignancies.¹⁴ Therefore mechanisms controlling the cellular distribution and expression of ECE-1 have attracted significant research interest. Previous approaches to study ECE-1 trafficking have involved immunoprecipitation,^{5,7} fluorescent antibodies⁵ and enzyme assays on membrane and cytosolic fractions of endothelial cells.¹⁰

Green fluorescent protein (GFP) is a widely used research tool to study the expression and trafficking of a large number of proteins. The first reported use of GFP in the field of endothelial biology relates to examining the effect of PKC induced phosphorylation on the distribution of ECE-1a and 1b isoforms.⁵ However, it has been speculated that the addition of molecular markers such as GFP and FLAG tags could influence the PKC mediated phosphorylation of ECE-1 therefore impacting on any conclusions drawn from experiments aimed at mapping ECE-1 distribution.⁵ We therefore examined the applicability of GFP to studies on ECE-1 trafficking. We have positioned a GFP tag at either the C (ECE-1c-GFP) or N (GFP-ECE-1c) terminus of ECE-1c and expressed in CHO-K1 cells. The effect of the GFP tag on catalytic activity and PMA induced phosphorylation was compared amongst the two chimeras and wild-type ECE-1c. Our results highlight that GFP can be applied to studies on ECE-1c phosphorylation and trafficking, only if placed at the C-terminal domain, although this modification results in a reduction of catalytic activity.

Results

Expression of GFP-tagged ECE-1c

GFP-tagged proteins were generated by adding GFP either to the N-terminal cytoplasmic tail (GFP-ECE-1c) or to the C-terminal extracellular (ECE-1c-GFP) domain of ECE-1c. Both GFP constructs were transfected in CHO-K1 cells and the expressed proteins were characterized. Both GFP tagged enzymes as well as the wild type was expressed in the cell mem-

brane (Fig. 1). Both GFP tagged proteins appeared larger than the wild type which corresponds to the addition of a GFP (27 KDa).

Effect of GFP tag on ECE-1c activity

The effect of a GFP tag placed at the N- or C-terminus of ECE-1c on catalytic activity was investigated by monitoring the cleavage of BigET₁₈₋₃₄ (a truncated version of the natural substrate Big Endothelin-1), by the membrane fractions of CHO-K1 cells expressing wild-type or GFP tagged ECE-1c. The specific cleavage of BigET₁₈₋₃₄ by ECE-1c results in the N- and C-terminal cleavage products of BigET₁₈₋₂₂ (P2; m/z 545; DIIW) and BigET₂₃₋₃₄ (P1; m/z 1380; VNTPEHVVPYGLG), respectively [Fig. 2(A)]. These products of cleavage were identified by MALDI-ToF mass spectrometry.

Both proteins (GFP-ECE-1c and ECE-1c-GFP) were catalytically active as evidenced by the cell membrane mediated cleavage of BigET₁₈₋₃₄, resulting in the expected N- and C-terminal cleavage products [Fig. 2(B,C)]. The relative amount of intact substrate left after incubation with membrane preparations for 3 h was determined by integrating the peak area corresponding to the substrate. Incubation of substrate with membrane preparation of cells transfected with GFP-ECE1c and wild-type ECE-1c resulted in 10% and 6% of intact substrate respectively [Fig. 2(A,B)]. However, incubation of substrate with membrane preparation of cells transfected with ECE-1c-GFP resulted in 67% of intact substrate after 3 h [Fig. 2(C)].

Effect of GFP on ECE-1c phosphorylation

Phosphorylation of ECE-1c was examined by immunoprecipitation using anti-ECE-1c anti-sera followed by autoradiography (Fig. 3). In agreement with previous studies^{6,10} stimulation of cells expressing wild-type ECE-1c with PMA resulted in a significant increase in ECE-1c phosphorylation (Fig. 4; $P < 0.02$; *significant compared to no PMA treatment

Figure 1. Expression of ECE-1c containing a GFP tag. Western blot of membrane and soluble fractions of wild-type ECE-1c (Lane 1), GFP-ECE-1c (Lane 2), and ECE-1c-GFP (Lane 3) transfected into CHO-K1 cells.

Figure 2. Effect of GFP tag on catalytic activity of ECE-1c. Catalytic activity was assessed by monitoring the cleavage of BigET₁₈₋₃₄ by reversed-phase HPLC analysis. Cells were transfected with (A) wild-type ECE-1c (B) GFP/ECE-1c and (C) ECE-1c/GFP. Peak P1 represents the C-terminal product (VNTPEHVVPYGLG; 1380 Da) after cleavage by ECE-1c, peak P2 the N-terminal product (DIIW; 545 Da) and peak S1 represents the intact peptide (DIIW VNTPEHVVPYGLG; 1907 Da). The chromatogram shown is representative of three independent experiments. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.interscience.wiley.com).]

Figure 3. A representative autoradiogram. Constitutive and PMA mediated (+) phosphorylation of wild-type ECE-1c (indicated by arrow) and GFP tagged ECE-1c (indicated by double arrow heads) in CHO-K1 cells.

in cells transfected with wild-type ECE-1c; unpaired *t*-test; $n = 4$). The addition of a GFP tag to the N or C-terminus of ECE-1c had no effect on the basal or constitutive phosphorylation of ECE-1c. The addition of GFP tag to the C-terminus continued to significantly increase the PMA induced phosphorylation of ECE-1c (Fig. 4; $**P < 0.04$; significant compared to no PMA treatment in cells transfected with ECE-1c-GFP; unpaired *t*-test; $n = 4$). The addition of a GFP tag to the N-terminus however abrogated the PMA induced increase in ECE-1c phosphorylation.

Confocal studies of GFP-tagged ECE-1c

The ECE-1c-GFP plasmid was titrated into CHO-K1 cells, and the transfected cells were observed 24 h after transfection. A DNA concentration of 100 ng/well which gave a maximum number of healthy cells expressing GFP-ECE-1c (data not shown) was chosen for further experiments. The cells were optically sectioned using confocal microscopy to reveal the intracellular localization of ECE-1c. Optical sectioning through the cell nucleus did not reveal any positive staining in this region for either of the GFP-constructs.

In non-treated CHO-K1 cells, ECE-1c-GFP (Fig. 5) failed to co-localize with Texas Red Agglutinin at the cell surface. The enzyme was restricted primarily to the cytoplasm with high aggregation next to the nucleus; consistent with an endoplasmic reticulum localization. Addition of PMA to the cells did not result in a plasma membrane localization. The protein did however, appear to be more homogeneously distributed within the cytoplasm resulting in less aggregated fluorescence.

In comparison GFP-ECE-1c displayed a high level of plasma membrane co-localization (Fig. 6). In addition, the enzyme did not appear to aggregate either toward the nucleus or below the plasma membrane, thus showing a weak, diffuse intracellular

Figure 4. Effect of GFP tag on phosphorylation of ECE-1c. Effect of a GFP tag on the N (GFP-ECE-1c) and C (ECE-1c-GFP) terminus of ECE-1c on PMA induced ECE-1c phosphorylation was examined. Results are expressed relative to untreated cells transfected with wild-type ECE-1c. The data are shown as the mean $\pm$ SEM from four separate experiments. $*P < 0.02$ versus untreated, $**P < 0.04$ versus untreated.

Figure 5. Confocal sections of CHO-K1 cells expressing ECE-1c-GFP. Cells were either unstimulated (–) or stimulated (+) with PMA (2 μ M, 10 min) before plasma membrane labeling with wheat-germ agglutinin-Texas Red conjugate to illuminate plasma membrane (left panels, using red channel). The middle panels show GFP-ECE-1c protein fluorescence using the “green channel.” Right panels are an overlay of the red and green images, with areas of membrane and ECE-1 co-localization appearing yellow. [$\times 100$ oil immersion (–) and $\times 63$ oil immersion (+)].

distribution. In some cells, the enzyme was exclusively at the cell surface. In PMA-treated cells, the GFP-ECE-1c remained almost exclusively at the plasma membrane.

Discussion

Production of ET-1 is dependent upon the expression of ECE-1 at the cell surface. Recent research has shown that phosphorylation is a key factor stimulat-

ing the trafficking of ECE-1 to the cell surface⁸ where it catalyzes the production of ET-1. Furthermore, ECE-1 can be shed from the cell surface to produce a soluble counterpart of the membrane bound form which retains catalytic activity.¹⁵ Since ET-1 and hence ECE-1 have been implicated in the pathogenesis of a range of different diseases,^{13,16–18} attention has been focused on the identification of the physiological or pathophysiological stimulants

Figure 6. Confocal sections of CHO-K1 cells expressing GFP-ECE-1c. Cells were either unstimulated (–) or stimulated (+) with PMA (2 μ M, 10 min) before plasma membrane labeling with a wheat-germ agglutinin-Texas Red conjugate to illuminate plasma membrane (left panels, using red channel). The middle panels show GFP-ECE-1c protein fluorescence using the “green channel.” Right panels are an overlay of the red and green images, with areas of membrane and ECE-1 co-localization appearing yellow ($\times 63$ oil immersion).

that induce ECE-1 phosphorylation and hence trafficking.² Previous research has used immunofluorescence⁵ and enzyme activity¹⁰ based approaches to study ECE-1 trafficking.

GFP is a widely used research tool to study the localization and trafficking of many proteins.¹⁹ GFP has been targeted successfully to every major organelle of the cell including the plasma membrane,²⁰ nucleus, and mitochondria.²¹ The size and shape of GFP and the different pHs and redox potentials found in the cell does not appear to restrict the potential uses of the GFP. ECE-1a and 1b isoforms containing a GFP tag have previously been expressed in CHO cells to study both basal and PMA induced phosphorylation of ECE-1.⁵ In this study, the authors raise the possibility that the GFP tag itself may alter the overall structure of ECE-1 making it more accessible for enzymes mediating phosphorylation.⁵ Some studies have also used ECE-1 containing a FLAG tag to study basal phosphorylation of ECE-1.⁷ It has been eluded that possible increase in phosphorylation was the result of the GFP tag itself, which may have led to inaccurate conclusions on enzyme distribution.⁵ This in turn leads to questions on the applicability of GFP in the study of ECE-1 distribution.

To address this issue, we transfected CHO-K1 cells with ECE-1c containing a GFP tag on the N or C-terminus. Comparison of the levels of phosphorylation between the two ECE-1c chimeras and wild type indicated that basal phosphorylation is insensitive to the GFP tag. However, the positioning of the GFP tag on the N-terminus inhibited PMA induced ECE-1c phosphorylation, while a C-terminal GFP tag had no effect on ECE-1c phosphorylation. Our data therefore support previous studies in which a GFP tag was placed at the C-terminus of ECE-1a and 1b to examine cellular distribution and trafficking in response to PMA.⁵

Catalytic activity of ECE-1c is confined to the C-terminal domain, while the only difference amongst the isoforms of ECE-1 is on the N-terminal cytoplasmic domain.^{3,4} This perhaps explains the insensitivity of ECE-1c catalytic activity to the N-terminal GFP tag. Our results clearly demonstrate that attaching GFP to the C-terminus results in a significant reduction of catalytic activity.

Despite the wide use of GFP, only a very few studies examine the potential artefacts associated with GFP tagging.²² A preliminary literature search failed to identify any studies examining the effect of differential localization of a GFP tag on enzyme activity and phosphorylation. Our study did not examine the mechanism(s) behind the reduced catalytic activity and phosphorylation in ECE-1c-GFP, and GFP-ECE-1c, respectively. However, it is highly likely that the addition of a 27 KDa GFP tag causes a conformational change or a steric hindrance in the

enzyme which prevents access to the substrate and PKC in ECE-1c-GFP, and GFP-ECE-1c, respectively. This would result in a reduction in catalytic activity in ECE-1c-GFP, and phosphorylation in GFP-ECE-1c. Determining the mechanism behind the reduced phosphorylation and catalytic activity in GFP-ECE-1c and ECE-1c-GFP, respectively, would require detailed studies taking structural approaches which are beyond the scope of this study. In addition, results from previous studies indicate that the use of GFP proteins to probe 3D conformational changes comes with limitations.²² These are related to the relatively large size of GFP tag (27 KDa) making it unsuitable for tagging small proteins. In addition, it has been reported that the N and/or C-terminus is not the optimum location a GFP tag to study protein conformational changes.

The cytoplasmic N-terminal domain of ECE-1 is a short peptide sequence of 35 amino acids with an approximate molecular mass of 5.5 KDa. The addition of a relatively large 27 KDa GFP tag to the N-terminus of ECE-1c is likely to impact on conformation and thus prevent access to PKC, resulting in the inability of the N-terminally tagged ECE-1c to be phosphorylated upon PKC activation. Previous studies by us have shown that Tyr4 and Ser35 residues in the N-terminus of ECE-1c are phosphorylated upon the activation of PKC. Mutation of either residue significantly inhibited PKC induced ECE-1c phosphorylation.⁸ It is likely the addition of the N-terminal GFP tag mainly inhibits phosphorylation of the Tyr4 residue reflecting its close proximity to the GFP tag.

The effects of GFP addition on ECE-1c localization was also analyzed. To differentiate between cell surface ECE-1c and intracellular enzyme, the plasma membrane of the cells was stained with wheat-germ agglutinin-Texas Red[®] conjugate, which binds to carbohydrates on the cell surface. Optical sections were therefore collected at two excitation wavelengths corresponding to GFP (green channel) and Texas Red (red channel). Co-localization was evidenced by a yellow colour upon overlaying the two images from red and green channels.

The two constructs did not localize similarly in non-stimulated CHO-K1 cells (Figs. 5 and 6). GFP-ECE-1c localized entirely within the cytoplasm in an aggregate, while the ECE-1c-GFP construct was almost entirely localized at the plasma membrane. The difference suggests that the addition of GFP can modify the enzyme trafficking in addition to phosphorylation and activity. Although the presence of ECE-1c-GFP in the plasma membrane was not observed following PMA stimulation, the protein appeared more equally distributed within the cytoplasm. This homogeneous distribution of ECE-1c-GFP was not observed with GFP-ECE-1c. The lack of a difference in the distribution of GFP-ECE-1c

following PMA stimulation is likely result of the inability of this chimera to be phosphorylated by PKC.

In conclusion, GFP is clearly a useful tool in the study of ECE-1c trafficking, however, the downstream applications of the GFP tagged ECE-1c must be considered when designing the constructs. The position of the GFP tag can effect the distribution of ECE-1c. Our data indicate that despite the reduction in catalytic activity, positioning the GFP tag on the C-terminal domain is optimal for studies aimed at monitoring the trafficking of ECE-1c via fluorescence microscopy. A GFP tag on the C-terminus has no effect on the phosphorylation of ECE-1c.

Methods

Cell culture

CHO-K1 cells were obtained from American Type Culture Collection (ATCC). The cells were maintained in media containing 50% α Mem, 40% Fetal Calf Serum and 10% DMSO.

Generation of green fluorescent protein tagged ECE-1c

A GFP tag was attached either to the N-terminal cytoplasmic tail or to the C-terminus of ECE-1c using protein expressing vectors. Both pEGFP-C1 and pEGFP-N1 were from Clontech (Palo Alto, CA).

A *Sma*I cut site was engineered by PCR at the 5' end of ECE-1c sequence to remove its end codon using the classical T7 primer and the following primer (GFP-*Sma*3: CCGCTCCCCCGGGACCAGAC TTCGCACTTGTGAGG). PCR product and GFP vectors were digested first with *Sma*I at 25°C for 3 h, followed by *Kpn*I at 37°C for a further 3 h according to the suppliers instructions (Promega: Madison). The reactions were stopped by the addition of loading dye. Samples were separated using 1% agarose in Tris-Borate-EDTA Buffer (TBE), and purified from gel slices using an In Gel Purification kit (Qiagen, Australia). Linearized DNA molecules (1:3 molar ratio of vector to insert) were ligated with T4 DNA ligase and incubated at 16°C overnight in ligation buffer. DH5 α bacterial cells (Gibco BRL; Rockville, MA) were then transformed by heat shock according to supplier's instructions, and potential clones sequenced for verification (Micromon, Monash University).

Expression and characterization of GFP tagged ECE-1c fusion proteins

Both constructs were expressed in CHO-K1 cells using the Lipofectamine transfection protocol previously described.⁸ But briefly, 1 μ g of DNA and 8 μ L of lipofectamine were diluted in a total of 200 μ L of OPTImem. The mixture was allowed to stand

for 20 min at room temperature. A further 800 μ L of OPTImem was then added bringing the final volume to 1 mL. After washing with 2 mL of OPTImem, each well in a 6-well sterile culture plate containing CHO-K1 cells (70% confluent) was treated with this mixture of DNA, Lipofectamine and OPTImem. After a 4–5 h incubation at 37°C, cells were grown in complete media for a further 24–36 h.

The GFP tagged ECE-1c mutants were characterized by Western immunoblotting, phosphorylation, and enzyme activity assays.

Membrane preparation

When confluent, cells were washed three times in warm Tris-buffered saline (TBS; 25 mM Tris-Cl; 150 mM NaCl; pH7.4), and scrapped off the plate in 2 mL ice-cold TBS. Cells were then sonicated three times at 5 s intervals using a Branson sonicator (Danbury, CT). The mixture was centrifuged at 70,000g (Beckman ultracentrifuge) at 4°C for 1 h. Pellets were then resuspended in TBS and centrifuged again under the same conditions. Soluble fraction (supernatant) was aliquoted and stored at –70°C until used. Pellets containing the membrane fraction was resuspended in 600 μ L of TBS per 10 mm tissue culture plates and stored at –70°C until used.

SDS PAGE and Western immunoblotting

For Western analysis, cells were lysed in RIPA buffer 36–48 h after transfection. Proteins were resolved on 8% SDS PAGE under reducing conditions and transferred on to a PVDF membrane. After blocking with 5% skim milk to prevent non specific binding, membrane was incubated with anti-serum against ECE-1c (1:5000) over night at 4°C. ECE-1c antiserum was raised in house (against ECE-1c Ile³⁷⁹-Pro⁵⁷⁷) using previously published methods.¹⁰ The immunoreactive bands were detected following incubation with appropriate secondary antibodies and enhanced chemiluminescent detection reagent.

Phosphorylation studies

Phosphorylation studies were performed as described previously.⁸ But briefly, once the transfected CHO-K1 cells were 70% confluent, they were serum starved for 16 h. Cells were then incubated with [³²P] Pi (80 μ Ci/well) in a phosphate free medium for 2 h, and stimulated with PMA (2 μ M) for 10 min at 37°C. After stimulation, cells were washed twice with ice-cold HBSS to stop trafficking of proteins to cell surface. Cell membranes were then solubilized with 0.3 mL/well of Ripa buffer. Cell lysates were harvested and the insoluble membrane fraction was removed by centrifugation. The supernatant was pre-cleared with protein A-agarose beads (10 μ L) in the presence of BSA (6%) at 4°C for at

least 1 h. The pre-cleared lysates was then incubated with protein A-agarose beads (20 μ L) and 2 μ L of serum containing ECE-1c antibodies over night at 4°C. The agarose beads were washed, and the immune precipitates resolved on 8% SDS-PAGE as described previously.⁸ Gels were fixed, dried, and exposed overnight against a Fuji-type BAS-III PhosphorImaging plate. The plates were subsequently read in a FUJIX Bio-imaging Analyser BAS 1000 and the data analyzed using MacBAS version 1.0.

ECE-1 activity assay

ECE-1c activity was measured based on the ability of the CHO-K1 cell lysates (obtained 36–48 h after transfection as described above) to cleave BigET_{18–34} (DIIWVNTPEHVVPYGLG, Auspep, Vic, Australia) a truncated version of the natural substrate. BigET_{18–34} (5 μ g; 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 four times the volume of 1% TFA in methanol. The samples were dried (Speed-Vac, Savant) and stored in –20°C until analyzed by HPLC and LC/MS for evidence of ECE-1c mediated substrate cleavage.

High performance liquid chromatography (HPLC)

Each dried sample reconstituted using 250 μ L of solvent A (0.08% TFA) was injected into a compressed 8 $\times$ 10 cm C18 Nova-pack reversed-phase column. Separation was achieved using a linear gradient (3–100%) of solvent B (70% acetonitrile and 0.08% TFA) over 30 min at a flow rate of 1 mL/min. Absorbance of emerging peaks was measured at 214 nm using a Waters HPLC system. The peptides corresponding to the emerging peaks were collected manually and identities determined by MALDI-ToF as described previously.¹⁵ The peak area corresponding to substrate was determined at each time point. Substrate peak area after 3 h was expressed as a % of the initial peak area, and taken as the relative amount of intact substrate remaining after 3 h.

Confocal microscopy

Twenty-four hours after transient transfection, CHO-K1 cells were trypsinized and seeded in an 8-well chamber slide at a density of 50,000 cells/well in a final volume of 500 μ L of α -MEM containing 10% FBS. After a further 24 h incubation at 37°C, the cells were washed and the media changed to serum free medium. The cells were incubated for a further 12 h before stimulation. Following PMA stimulation (10 min, 2 μ M), cells were fixed in 0.5 mL of 4% paraformaldehyde in sodium phosphate buffer, pH 7.4 for 20 min at room temperature. Cells were washed in stimulating buffer (1% BSA, pre-warmed HBSS) and incubated in blocking

buffer (FBS, glycine and stimulating buffer) for 10 min at room temperature. Cells were washed again before incubation in wheat-germ agglutinin/Texas Red[®] solution (2 μ g/mL) for 30 min. Cells were fixed in paraformaldehyde for 20 min. Chambers were removed from the slides, which were then washed twice in cold HBSS before coverslip mounting with 90% glycerol/10% HBSS.

Cells were viewed with a confocal laser scanning microscope (Bio-Rad MRC1024-Zeiss Axioscop) equipped with a Krypton/Argon laser source, 100 $\times$ oil immersion objective and dual-channel photodetectors. Optical sections were collected at excitation wavelengths of 488 and 568 nm to image the distribution of GFP-tagged proteins and Texas red-labeled cell surface. Images were scanned and line averaged 3 or 10 times to obtain a high signal-to-noise ratio. Laser intensity was kept constant for each experiment (3, 10, or 30% power). Colocalization of double-labeled proteins was determined by electronic overlay of signal obtained from Channel A (red channel) and B (green channel) and images were collected by Lasersharp Acquisition software.

Statistical analysis

For all comparisons, data were analyzed by unpaired student's *t*-test. All data are presented as Mean $\pm$ SEM and a value of *P* < 0.05 was considered to be significantly different.

References

- Ohnaka K, Takayanagi R, Nishikawa M, Haji M, Nawata H (1993) Purification and characterization of a phosphoramidon-sensitive endothelin-converting enzyme in porcine aortic endothelium. *J Biol Chem* 268:26759–26766.
- Kuruppu S, Smith AI (2012) Endothelin converting enzyme-1 phosphorylation and trafficking. *FEBS Lett* 586:2212–2217.
- Schweizer A, Valdenaire O, Nelbock P, Deuschle U, Dumas Milne Edwards JB, Stumpf JG, Löffler BM (1997) Human endothelin-converting enzyme (ECE-1): three isoforms with distinct subcellular localizations. *Biochem J* 328:871–877.
- Valdenaire O, Lepailleur-Enouf D, Egidy G, Thouard A, Barret A, Vranckx R, Tougard C, Michel JB (1999) A fourth isoform of endothelin-converting enzyme (ECE-1) is generated from an additional promoter molecular cloning and characterization. *Eur J Biochem/FEBS* 264:341–349.
- Jafri F, Ergul A (2006) Phosphorylation of endothelin converting enzyme-1 isoforms: relevance to subcellular localization. *Exp Biol Med* 231:713–717.
- Smith AI, Lew RA, Thomas WG, Tochon-Danguy N (2006) Protein kinase C regulates the cell surface activity of endothelin-converting enzyme-1. *Int J Pept Res Ther* 12:291–295.
- MacLeod KJ, Husain RD, Gage DA, Ahn K (2002) Constitutive phosphorylation of human endothelin-converting enzyme-1 isoforms. *J Biol Chem* 277:46355–46363.
- Kuruppu S, Tochon-Danguy N, Smith AI (2012) Protein kinase C recognition sites in the cytoplasmic domain of

- endothelin converting enzyme-1c. *Biochem Biophys Res Commun* 427:606–610.
9. Keynan S, Khamaisi M, Dahan R, Barnes K, Jackson CD, Turner AJ, Raz I (2004) Increased expression of endothelin-converting enzyme-1c isoform in response to high glucose levels in endothelial cells. *J Vasc Res* 41: 131–140.
 10. Kuruppu S, Tochon-Danguy N, Ian Smith A (2010) Role of protein kinase C in endothelin converting enzyme-1 trafficking and shedding from endothelial cells. *Biochem Biophys Res Commun* 398:173–177.
 11. Jeng AY (2003) Utility of endothelin-converting enzyme inhibitors for the treatment of cardiovascular diseases. *Curr Opin Investig Drugs* 4:1076–1081.
 12. Martinez-Miguel P, Raach V, Zaragoza C, Valdivielso JM, Rodriguez-Puyol M, Rodriguez-Puyol D, Lopez-Ongil S (2009) Endothelin-converting enzyme-1 increases in atherosclerotic mice: potential role of oxidized low density lipoproteins. *J Lipid Res* 50:364–375.
 13. Pluta RM, Boock RJ, Afshar JK, Clouse K, Bacic M, Ehrenreich H, Oldfield EH (1997) Source and cause of endothelin-1 release into cerebrospinal fluid after subarachnoid hemorrhage. *J Neurosurg* 87:287–293.
 14. Smollich M, Wulfig P (2008) Targeting the endothelin system: novel therapeutic options in gynecological, urological and breast cancers. *Exp Rev Anticancer Ther* 8: 1481–1493.
 15. Kuruppu S, Reeve S, Ian Smith A (2007) Characterisation of endothelin converting enzyme-1 shedding from endothelial cells. *FEBS Lett* 581:4501–4506.
 16. Morawietz H, Duerschmidt N, Niemann B, Galle J, Sawamura T, Holtz J (2001) Induction of the oxLDL receptor LOX-1 by endothelin-1 in human endothelial cells. *Biochem Biophys Res Commun* 284:961–965.
 17. Rosano L, Spinella F, Di Castro V, Decandia S, Nicotra MR, Natali PG, Bagnato A (2006) Endothelin-1 is required during epithelial to mesenchymal transition in ovarian cancer progression. *Exp Biol Med* 231:1128–1131.
 18. Smollich M, Gotte M, Yip GW, Yong ES, Kersting C, Fischgrabe J, Radke I, Kiesel L, Wulfig P (2007) On the role of endothelin-converting enzyme-1 (ECE-1) and neprilysin in human breast cancer. *Breast Can Res Treat* 106:361–369.
 19. Tavares JM, Fletcher LM, Welsh GI (2001) Using green fluorescent protein to study intracellular signalling. *J Endocrin* 170:297–306.
 20. Hanakam F, Albrecht R, Eckerskorn C, Matzner M, Gerisch G (1996) Myristoylated and non-myristoylated forms of the pH sensor protein hisactophilin II: intracellular shuttling to plasma membrane and nucleus monitored in real time by a fusion with green fluorescent protein. *EMBO J* 15:2935–2943.
 21. Rizzuto R, Brini M, De Giorgi F, Rossi R, Heim R, Tsien RY, Pozzan T (1996) Double labelling of subcellular structures with organelle-targeted GFP mutants in vivo. *Curr Biol* 6:183–188.
 22. Viallet PM, Vo-Dinh T (2003) Monitoring intracellular proteins using fluorescence techniques: from protein synthesis and localization to activity. *Curr Prot Peptide Sci* 4:375–388.