Anti-peptide antibodies specific to rat endothelin-converting enzyme-1 isoforms reveal isoform localisation and expression

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Abstract Endothelin-converting enzyme-1 (ECE-1) is a critical enzyme in the biosynthesis of the potent vasoconstrictor peptide endothelin and exists in several isoforms. Anti-peptide antibodies raised against epitopes in the distinct N-terminal cytoplasmic tails of the rat ECE-1 isoforms have been obtained. By using these antibodies in Western blot analysis and immunofluorescence studies, we have shown that cultures of transformed rat lung vascular endothelial cells treated with the metalloprotease inhibitor phosphoramidon and untreated cells express ECE-1α only, whereas human umbilical vein endothelial cells express ECE-1α and ECE-1β. The ECE-1 isoforms expressed in CHO-K1 cells transfected with rat cDNA to ECE-1α and ECE-1β could be immunoprecipitated by using the appropriate isospecific antibody.

Key words: Endothelin; Converting enzyme; Immunofluorescence; Immunoprecipitation; Isoform; Endothelial cell

1. Introduction

The endothelins were first isolated from the supernatant of cultured endothelial cells [1]. Endothelins 1, 2 and 3 (ET-1, ET-2 and ET-3) are vasoactive peptides which are synthesised from proprotein precursors via the constitutive secretory pathway. Endothelin-converting enzyme (ECE) is unique to pro-endothelin processing in endothelial, smooth muscle and other cell types and its proteolytic action produces ET-1 from its inactive intermediate peptide, big-ET-1. ECE is, therefore, a potentially important therapeutic target for cardiovascular and other diseases (for review see [2]).

ECE was first purified to homogeneity from rat lung [3]. Cloning of the rat, human and bovine cDNAs to ECE-1 rapidly followed [4–8]. The ECE-1 polypeptides from these species were found to have a high sequence similarity to each other, except at their extreme N-termini. The presence of two isoforms of ECE-1 was recognised in rat and these were termed ECE-1α and ECE-1β (and in human ECE-1b and ECE-1a, respectively) according to their NH_2-terminal sequences [9] [10]. The isoforms were found to be identical in specific activity and substrate specificity [9–11]. The two isoforms are encoded by the same gene and are generated through the use of alternative promoters [10]. Although differences in the relative mRNA expression ratios of the ECE-1 isoforms have been detected, rat and human ECE-1α mRNA was usually found to be the more abundant form in the various tissues and cells examined [9] [10]. In addition, an apparent increase in ECE-1 protein levels, but not in ECE-1 mRNA levels, by the ECE metalloprotease inhibitors phosphoramidon and thiorphan has been noted previously [5] [12]. To date, however, it has not been possible to measure the relative levels of protein expression of the two isoforms. Although antibodies which recognise the C-terminal domain of rat and human ECE-1 have been used extensively in immunological studies [12–14], these antibodies do not distinguish the ECE-1α and ECE-1β proteins. We have now developed polyclonal antibodies to the N-termini of ECE-1α and ECE-1β which are able to distinguish immunologically between the two proteins. We have exploited these antibodies to evaluate ECE-1 protein expression in cell lines. The antibodies are suitable for use in immunofluorescence, Western blotting and immunoprecipitation.

2. Materials and methods

2.1. Materials

CHO cells were obtained from the European Collection of Cell Culture (Salisbury, Wiltshire, UK). Simian virus 40-transformed rat lung vascular endothelial cells (TRLEC-03 cells) were donated by Dr. S. Tsurufuji, Institute of Cytosignal Research, Tokyo, Japan, and human umbilical vein endothelial cells (HUVEC) were obtained as cryopreserved primary cultures from Clonetics, TCS Biologicals Ltd, Buckingham, UK. Penicillin, streptomycin, non-essential amino acids, foetal calf serum, trypsin/EDTA and transfection reagent (lipofectAmine) were purchased from Gibco-BRL (Paisley, UK). Glasgow minimal essential medium, nucleosides and sodium pyruvate were purchased from Sigma (Poole, Dorset, UK). The plasmid pcDLSRα296/rECE (containing rat ECE-1α or ECE-1β) was a kind gift from Dr. K. Tanzawa (Sankyo, Tokyo, Japan) [6]. Synthetic peptides were purchased from Genosys Biotechnologies Inc., Texas. Sulfo MBS and Sulfolink gel were obtained from Pierce (Rockford, IL, USA). The ECL Western blotting kit was obtained from Amersham International plc.

2.2. Antibody production

Peptides A (SYKRATLDEEDLC; ECE-1α), B (SLRPPQGLGLQWSSC; ECE-1β) and C (CLGKKGPGLTVSLPL; ECE-1β) were coupled via a C-terminal cysteine (peptides A and B) or an N-terminal cysteine (peptide C) to ovalbumin using standard procedures (Fig. 1) [15]. New Zealand White rabbits were immunised with the conjugated peptides according to standard protocols [15]. A preimmune bleed was taken from each rabbit before immunisation. 200 μg of peptide was injected initially and 100 μg in subsequent injections every 4 weeks. Sample bleeds were taken 7–10 days after each immunisation to obtain the peak response. Serum was stored at −70°C.

Specificity and sensitivity of antibody production was determined by enzyme-linked immunosorbent assay (ELISA) using standard methods [15]. The secondary antibody (anti-rabbit horseradish peroxidase IgG) was diluted 1/1000. The substrate used was 2,2′-azino-

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Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); CHO, Chinese hamster ovary; ECE, endothelin converting enzyme; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; HUVEC, human umbilical vein endothelial cells; TRLEC-03, transformed rat lung vascular endothelial cells

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bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in 5.72 ml 0.1 M citric acid, 4.27 ml 0.2 M Na_2HPO_4, 1 μl 30% H_2O_2, pH 4.3. Assays were also carried out in the absence of antisera and with preimmune serum replacing antiserum. Specificity of the antisera was assayed by binding to the appropriate opposing peptide.

Antibodies were purified by using an affinity column prepared by the attachment of the cysteine containing peptides to iodoacetyl-agarose (Sulfolink) according to the manufacturer's recommendations. The specific antibodies were eluted with diethylamine, pH 11, or glycine-HCl, pH 2.4. Elution of the antibodies from the column was confirmed by ELISA as described above.

2.3. Gel electrophoresis and blotting

SDS-PAGE sample buffer was added to the samples which were then treated with 5% β-mercaptoethanol and subjected to SDS-PAGE using a 7.5% resolving gel [12]. Western blotting was carried out by using the Amersham enhanced chemiluminescence (ECL) kit and protocol. The purified anti-peptide antibodies against rat ECE-1α and ECE-1β were diluted 1/500. The secondary antibody was the anti-rabbit horseradish peroxidase conjugate (dilution 1/5000).

2.4. Cell culture

CHO-K1 cells were cultured in Glasgow minimal essential medium containing 10% (v/v) foetal calf serum as described in [16]. TRLEC-03 cells were cultured in RPMI-1640 medium containing 10% (v/v) foetal calf serum and HUVECs were grown in a supplemented endothelial basal medium, according to the suppliers' instructions. All cells were grown in a humidified incubator in a 5% CO_2 atmosphere at 37°C. Membrane fractions were prepared from cells as described previously [17].

2.5. Immunofluorescence

TRLEC-03, HUVEC and CHO-K1 cells were grown on coverslips in 24-well plates. CHO-K1 cells were transiently transfected with rat ECE-1α or ECE-1β [16] and the cells were fixed and stained 24–48 h post-transfection. The TRLEC-03, HUVEC and CHO-K1 cells were fixed with methanol/acetone (1:1) and immunostained as in [12]. The purified anti-peptide antibodies were diluted 1/50 (anti-ECE-1α; AS-66) or 1/100 (anti-ECE-1β; AS-65). The secondary antibody was anti-rabbit fluorescein isothiocyanate (FITC) (1/100).

2.6. Immunoprecipitation

A sample of a 1 mg/ml solubilised membrane preparation of ECE-1α or ECE-1β transfected CHO cells was preincubated with protein A (10% v/v) for 1 h at 4°C with rotation, followed by centrifugation at 10 000×*g* for 15 s at 4°C. Preimmune serum (0.5 μl) and each of the anti-peptide antibodies were separately incubated with a 1 ml sample of the supernatant for 1 h on ice. Protein A was then added to each sample (10% v/v) and incubated for 1 h at 4°C with rotation. The beads were collected by centrifugation at 10 000×*g* for 15 s at 4°C and washed with Tris-buffered saline (50 mM Tris, 150 mM sodium chloride, pH 7.4) containing 0.1% (v/v) Tween 20 (TBST). Sample buffer (20 μl) was added and the samples were heated to 85°C for 10 min. After centrifugation (10 000×*g*, 15 s, 4°C), 1 μl β- mercaptoethanol was added to each supernatant which was then heated to 100°C for 4 min. The samples were subjected to SDS-PAGE on a 7.5% gel as described previously and blotted using the ECL system. The primary antibodies were diluted 1/500.

ECE 1-α

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rat      MMSSYKRATLDEEDLVDSLSEGDVYPNG---
human     ..T..................A....---
bovine   ...T...P.............S.....H---
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ECE 1-β

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rat      MGSLRPPQGLGLQWSSFFLGKKGPGLTVSLPLLASS-
human       M.L......RNP.LQ..R.....S.P...PP.-
bovine      MS.R.QD.LR.PLL..SEA....S.PFR.PP.-
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Fig. 1. Comparison of the N terminal sequences of ECE-1α and ECE-1β of rat, human and bovine cDNA. Underlined regions indicate the sequences in rat cDNA from which peptides were designed to raise specific anti-ECE-1α and anti-ECE-1β antibodies. ECE-1α: peptide A, SYKRATLDEEDLC; ECE-1β: peptide B, SLRPPQGLGLQWSSC; ECE-1β: peptide C, CLGKKGPGLTVSLPL.

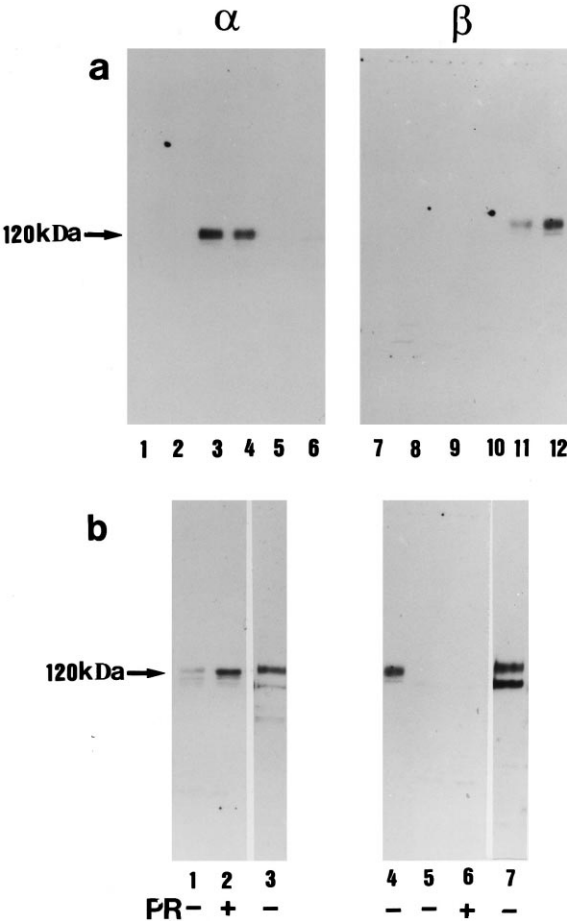

Fig. 2. Immunoblots of ECE-1α and ECE-1β expressed in CHO-K1 cells. a: Untransfected cells (lanes 1 and 2, 7 and 8), ECE-1α transfected cells (lanes 3 and 4, 9 and 10) and ECE-1β transfected cells (lanes 5 and 6, 11 and 12) were blotted with AS-66 to ECE-1α (α) and AS-65 to ECE-1β (β). Lanes 1, 3, 5, 7, 9 and 11 were loaded with 6 μg of protein. Lanes 2, 4, 6, 8, 10 and 12 were loaded with 10 μg of protein. b: Immunoblots of ECE-1 in TRLEC-03 and HUVEC cells. Lanes 1 and 5 were loaded with 15 μg of protein from untreated TRLEC-03 cells. Lanes 2 and 6 were loaded with 15 μg of protein from TRLEC-03 cells treated with phosphoramidon (PR). Lane 4 was loaded with 10 μg of protein from ECE-1β transfected CHO cells. Lanes 3 and 7 were loaded with 10 μg of protein from HUVEC membranes. The samples were blotted with AS-66 to ECE-1α (α) and AS-65 to ECE-1β (β).

3. Results

3.1. ELISA

ELISA revealed that the antisera against peptides A, B and C showed immunoreactivity with the corresponding peptides. The antibodies AS-66 to ECE-1α (raised against peptide A) and AS-65 to ECE-1β (raised against peptide C) showed the greatest immune response, being 15–20 times that of the preimmune bleed. These two antisera were, therefore, chosen for purification. The antiserum against peptide B showed a weak response (2–3 times that of the preimmune serum) and was

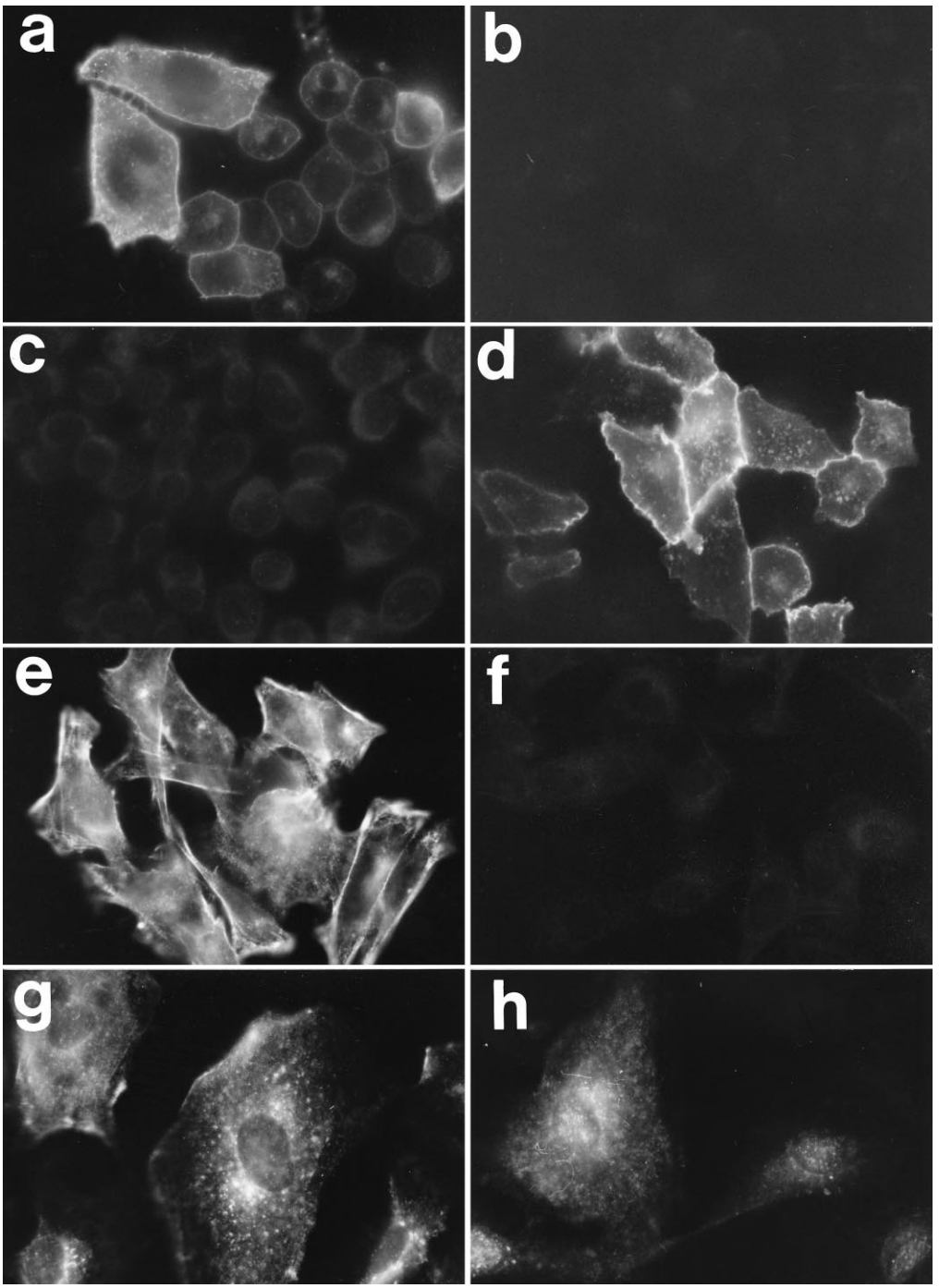

Fig. 3. Immunofluorescent microscopy of ECE-1 using isoform-specific antibodies. Immunofluorescence of ECE-1α transfected CHO cells (a and b), ECE-1β transfected CHO cells (c and d), TRLEC-03 cells (e and f) and HUVEC cells (g and h) was carried out by using AS-66 to ECE-1α (left-hand panel) and AS-65 to ECE-1β (right-hand panel). For details see Section 2.

therefore not purified or characterised further. Serial dilutions of each antiserum were evaluated by ELISA and the relevant dilution appropriate for subsequent studies was determined (results not shown).

3.2. Western blotting

CHO cells were transfected with either rat ECE-1α or ECE-1β cDNA. Western blotting of membranes prepared from these cells, using the purified antisera AS-66 and AS-65, revealed that each antiserum recognised the corresponding protein specifically (Fig. 2a, lanes 3 and 4, 11 and 12). No cross-reactivity was observed with untransfected CHO cells (Fig. 2a, lanes 1 and 2, 7 and 8) or with CHO cells expressing the opposing ECE-1α or ECE-1β protein (Fig. 2a, lanes 5 and 6, 9 and 10).

Immunoblotting of TRLEC-03 membranes indicated the presence of ECE-1α at an M_r of 120 000 (Fig. 2b, lane 1), the level of which was increased approximately 3-fold (as

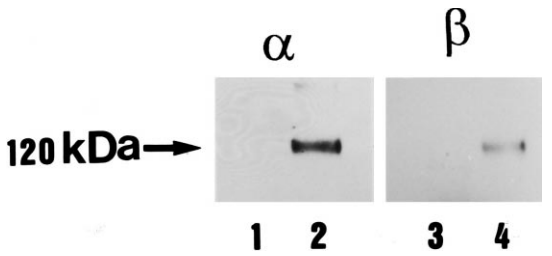

Fig. 4. Immunoprecipitation of ECE-1 by the isoform-specific anti-ECE antibodies. Solubilised membranes from CHO cells transfected with the respective rat ECE-1 isoform cDNAs were incubated with the relevant antibody. The antibody complex was then immunoprecipitated by treatment with protein A. In one sample, preimmune serum was used as a control. Lanes 1 and 3 show treatment with the preimmune serum and lanes 2 and 4 show immunoprecipitation of ECE-1α and ECE-1β by AS-66 and AS-65, respectively.

quantified by densitometry (Scanmaster 3, Howtek)) following treatment of the cells with phosphoramidon (Fig. 2b, lane 2). Although ECE-1β was not detected in the TRLEC-03 membranes (Fig. 2b, lanes 5 and 6), membranes from CHO cells transfected with rat ECE-1β were immunopositive for this isoform (Fig. 2b, lane 4) with a similar M_r. By way of contrast, in HUVEC membranes both isoforms were present, ECE-1β (Fig. 2b, lane 7) being more abundant than ECE-1α (Fig. 2b, lane 3). The antibodies, although designed against the rat sequences, are therefore suitable for detection of the corresponding human ECE-1 isoforms. The lower immunoreactive band (M_r 97 000) seen in each lane has been observed previously in HUVECs when using the monoclonal antibody AEC 32-236 and its presence is probably due to proteolysis [7,12].

3.3. Immunofluorescence

CHO-K1 cells transiently transfected with rat ECE-1α or ECE-1β, TRLEC-03 cells and HUVEC cells were analysed by immunofluorescent microscopy using the isoform specific antibodies AS-66 (Fig. 3, left-hand panel) and AS-65 (Fig. 3, right-hand panel). Staining was observed when CHO cells expressing ECE-1α or ECE-1β were incubated with their corresponding antibody (Fig. 3a,d, respectively). By way of contrast, ECE-1α or ECE-1β transfected CHO cells showed no staining when incubated with the opposing antibodies (Fig. 3b,c, respectively). In transfected CHO cells, ECE-1α and ECE-1β both showed a predominantly plasma membrane location, as did the ECE-1α detected in TRLEC-03 cells.

As with the Western blots, staining was apparent in TRLEC-03 cells incubated with the ECE-1α antibody, but no staining was seen with the ECE-1β antibody in these cells (Fig. 3e,f). Staining of both isoforms was revealed in HUVEC cells incubated with either AS-66 or AS-65 (Fig. 3g,h).

3.4. Immunoprecipitation

The antibodies AS-66 and AS-65 were incubated with solubilised membranes from CHO cells transfected with their respective ECE-1 isoform cDNAs on ice for 1 h. Immunoprecipitation was then carried out by binding of the immune complexes to protein A. Preimmune serum replaced the antiserum in one sample as a control. SDS-PAGE of these samples followed by Western blotting for ECE-1, using the isoform-specific antibodies as the primary antibodies in each case, revealed that both antibodies immunoprecipitated the relevant corresponding protein (Fig. 4, lanes 2 and 4). No ECE-1 was detected in the control samples where preimmune serum replaced the anti-peptide antibodies (Fig. 4, lanes 1 and 3).

4. Discussion

In this study, we describe for the first time the production and characterisation of polyclonal antibodies raised to peptides contained within the N-terminal cytoplasmic domains of the ECE-1α and ECE-1β isoforms. Immunofluorescence studies and Western blot analysis of ECE-1 expressed in CHO-K1 cells demonstrate that the antibodies AS-66 and AS-65 stain specifically the ECE-1α and ECE-1β proteins, respectively. Previously described monoclonal antibodies raised to ECE-1 [13] recognise epitopes in the C-terminal domain and therefore bind both isoforms. As these monoclonal antibodies are unable to discriminate between the proteins, determination of the distribution and expression levels of the isoforms in various tissues and cells has not previously been possible.

Here, we demonstrate for the first time, by immunofluorescence and Western blot analysis, the protein expression of both ECE-1 isoforms in HUVEC cells and also establish that only the ECE-1α isoform is expressed in TRLEC-03 cells. Previous observations have shown an increase in the level of ECE-1 following treatment of TRLEC-03 cells with metalloprotease inhibitors, especially phosphoramidon [5,12]. However, at that time, the existence of ECE-1 isoforms was not recognised. This increased level of ECE-1 has now been unequivocally identified as the α isoform with the novel isospecific antibodies.

The level of isoform expression may be of significance in different pathological states and this variation in protein expression could be used as an indicator of disease progression and/or efficacy of therapeutic intervention. For example, the increase in level of ECE-1 following balloon injuries in rat carotid arteries, used as a model for human atherosclerotic lesions [18], could be a reflection of an increase in both or only one of the isoforms. The availability of isoform-specific antibodies could therefore be of value in such clinical investigations. Relative expression levels of the ECE-1 mRNAs may also be affected by cell transformation. For example, it is possible that the transformation of rat lung endothelial cells with SV-40 DNA resulted in down-regulation of ECE-1β mRNA, thus explaining the detection of ECE-1α only in this cell line. Since ECE-1α and ECE-1β have different tissue distributions and expression levels, this may be a reflection of distinct physiological roles.

In contrast to the monoclonal antibodies to ECE-1 produced previously which did not immunoprecipitate the corresponding protein [13], immunoprecipitation of the respective ECE-1 isoforms is possible using the polyclonal antibodies AS-66 and AS-65. These antibodies could be useful for further characterisation of ECE-1, determination of the distribution of the isoforms both at the subcellular level in cells and in tissues, and in the clarification of the role of the ECE-1 isoforms in various pathological states.

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