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Identification of mouse orthologue of endogenous secretory receptor for advanced glycation end-products: structure, function and expression

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The cell-surface RAGE [receptor for AGE (advanced glycation end-products)] is associated with the development of diabetic vascular complications, neurodegenerative disorders and inflammation. Recently, we isolated a human RAGE splice variant, which can work as a decoy receptor for RAGE ligands, and named it esRAGE (endogenous secretory RAGE). In the present study, we have isolated the murine equivalent of esRAGE from brain polysomal poly(A)$^+$ (polyadenylated) RNA by RT (reverse transcription)–PCR cloning. The mRNA was generated by alternative splicing, and it encoded a 334-amino-acid protein with a signal sequence, but lacking the transmembrane domain. A transfection experiment revealed that the mRNA was actually translated as deduced to yield the secretory protein working as a decoy in AGE-induced NF-κB (nuclear factor κB) activation. RT–PCR and immunoblotting detected esRAGE mRNA and protein in the brain, lung, kidney and small intestine of wild-type mice, but not of RAGE-null mice. The esRAGE expression was increased in the kidney of diabetic wild-type mice. The present study has thus provided an animal orthologue of esRAGE for clarification of its roles in health and disease.

Key words: advanced glycation end-product, endogenous secretory receptor for advanced glycation end-products (esRAGE), immunohistochemistry, reverse transcription–PCR cloning.

INTRODUCTION

The RAGE [receptor for AGE (advanced glycation end-products)] belongs to an immunoglobulin superfamily of cell-surface receptors. It is composed of an N-terminal extracellular domain with a ligand-engaging V-region-like domain and two C-region-like domains, a single-pass transmembrane domain and a C-terminal highly charged short cytoplasmic domain, which is essential for signal transduction [1–4]. RAGE is known to participate in physiological and pathological processes, such as diabetic complications, atherosclerosis, inflammation and neurodegenerative disorders. We have also demonstrated that RAGE overexpression dramatically accelerated diabetic kidney changes in mice [5].

Previously, a soluble form of RAGE (sRAGE) produced by recombinant gene technology has been shown to exert a therapeutic potential in experimental diabetic complications. For instance, sRAGE dose-dependently suppressed the development of atherosclerotic lesions in diabetic apoE-null mice [6]; sRAGE-treated *db*/*db* mice displayed diminished albuminuria and glomerulosclerosis [7]. More recently, we identified a naturally occurring soluble RAGE in humans and named it endogenous secretory RAGE (esRAGE) [8]. esRAGE is generated by alternative splicing, and captures AGE ligands, thereby protecting cells from AGE-induced injury [8]. Immunohistochemical analysis with RAGE domain-specific antibodies indicated that esRAGE may be a predominant form of RAGE protein in a variety of human tissues and organs, such as the brain, kidney and intestine [9].

To clarify further the physiological and pathological roles of esRAGE, we considered it necessary to have an animal model in which its equivalent can be produced. As such, the present study was conducted to identify the murine homologue of the AGE-engaging decoy receptor.

EXPERIMENTAL

Mice

Male RAGE-null mice [10] backcrossed into the C57BL/6J strain (F7) and their wild-type counterparts at 16 weeks of age were used for RT (reverse transcription)–PCR and Western blotting. The protocol was approved by the Institutional Animal Care and Use Committee of Kanazawa University.

cDNA cloning

Polysomal poly(A)$^+$ (polyadenylated) RNA was isolated from the brain of a C57BL/6J wild-type mouse and was reverse-transcribed as described previously [8]. cDNA was initially amplified with 5′- and 3′-primers (5′-CACCATGCCAGCGGGGAC-3′ and 5′-AGCTCTGCACGTTCCTCCTCAT-3′) (nucleotides 2–19 and 1144–1165 respectively of GenBank® accession number L33412; F1 and R1 in Figure 1A) that corresponded to exons 1 and 11 of the mouse RAGE gene respectively, and then with 5′- and 3′-primers (5′-GTTCTTGCTCTATGGGGAGC-3′ and 5′-CACATGCGGCAGCCATAT-3′) (nucleotides 74298–74313 and 74509–74512,

Abbreviations used: AGE, advanced glycation end-products; DMEM, Dulbecco's modified Eagle's medium; esRAGE, endogenous secretory receptor for AGE; FBS, foetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NF-κB, nuclear factor κB; poly(A)$^+$, polyadenylated; RAGE, receptor for AGE; RT, reverse transcription; RU, response units; SPR, surface plasmon resonance; sRAGE, soluble form of RAGE.

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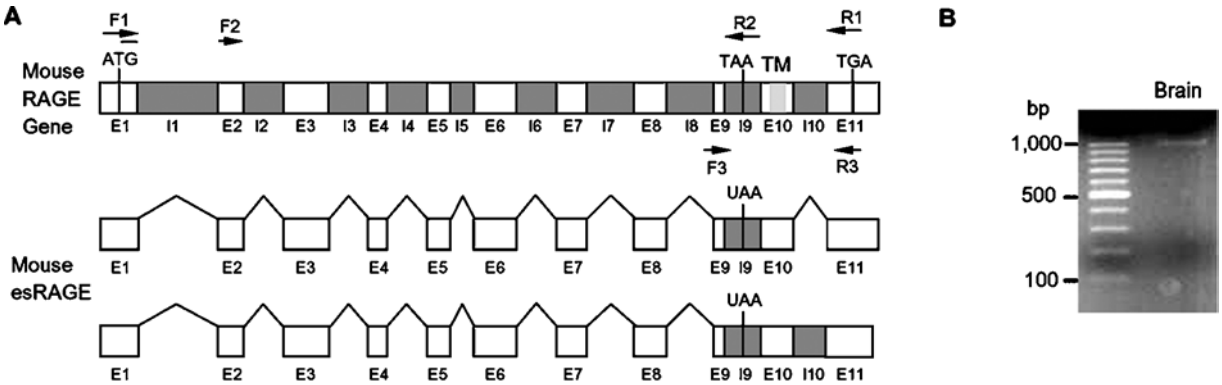
The nucleotide sequence data reported for mouse endogenous secretory receptor for advanced glycation end-products appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AB207883.



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Mouse full RAGE   1 : MPAGTAARAWVLVLALWGAVAGGQNITARIGEPLVLSCKGAPKKPPQQLEWKLNTGRTEA   60
                      ************************************************************
Mouse esRAGE      1 : MPAGTAARAWVLVLALWGAVAGGQNITARIGEPLVLSCKGAPKKPPQQLEWKLNTGRTEA   60
                      * ****  ****** ***** * ************* ********** ************
Human esRAGE      1 : MAAGTAVGAWVLVLSLWGAVVGAQNITARIGEPLVLKCKGAPKKPPQRLEWKLNTGRTEA   60

Mouse full RAGE  61: WKVLSPQGG-PWDSVAQILPNGSLLLPATGIVDEGTFRCRATNRRGKEVKSNYRVRVYQI   120
                      ************************************************************
Mouse esRAGE      61: WKVLSPQGG-PWDSVAQILPNGSLLLPATGIVDEGTFRCRATNRRGKEVKSNYRVRVYQI   120
                      ********* ******  ****** *** ** *** *** * ** *** ************
Human esRAGE      61: WKVLSPQGGGPWDSVARVLPNGSLFLPAVGIQDEGIFRCQAMNRNGKETKSNYRVRVYQI   120

Mouse full RAGE  121: PGKPEIVDPASELTASVPNKVGTCVSEGSYPAGTLSWHLDGKLLIPDGKETLVKEETRRH   180
                      ************************************************************
Mouse esRAGE      121: PGKPEIVDPASELTASVPNKVGTCVSEGSYPAGTLSWHLDGKLLIPDGKETLVKEETRRH   180
                      ************** ************************* * *  *   *** ****
Human esRAGE      121: PGKPEIVDSASELTAGVPNKVGTCVSEGSYPAGTLSWHLDGKPLVPNEKGVSVKEQTRRH   180

Mouse full RAGE  181: PETGLFTLRSELTVIPTQGGTTHPTFSCSFSLGLPRRRPLNTAPIQLRVREPGPPEGIQL   240
                      ************************************************************
Mouse esRAGE      181: PETGLFTLRSELTVIPTQGGTTHPTFSCSFSLGLPRRRPLNTAPIQLRVREPGPPEGIQL   240
                      ******** *** * *  **   ******** **** * * ** ** ** ** * *  **
Human esRAGE      181: PETGLFTLQSELMVTPARGGDPRPTFSCSFSPGLPRHRALRTAPIQPRVWEPVPLEEVQL   240

Mouse full RAGE  241: LVEPEGGIVAPGGTVTLTCAISAQPPPQVHWIKDGAPLPLAPSPVLLLPEVGHADEGTYS   300
                      ************************************************************
Mouse esRAGE      241: LVEPEGGIVAPGGTVTLTCAISAQPPPQVHWIKDGAPLPLAPSPVLLLPEVGHADEGTYS   300
                       ****** ***********   *** ** ** *** **** ***** *** *  * ****
Human esRAGE      241: VVEPEGGAVAPGGTVTLTCEVPAQPSPQIHWMKDGVPLPLPPSPVLILPEIGPQDQGTYS   300

                                                    ▼
Mouse full RAGE  301: CVATHPSHGPQESPPVSIRVTETGDEGPAEGSVGESGLGTLALALGILGGLGVVALLVGAI   360
                      ******************************
Mouse esRAGE      301: CVATHPSHGPQESPPVSIRVTETGDEGPAEGEGLD   334
                      ***** *******  **    * * ***  *** *
Human esRAGE      301: CVATHSSHGPQESRAVSISIIEPGEEGPTAGEGFDKVREAEDSPQHM   347

Mouse full RAGE  361: LWRKRQPRREERKAPESQEDEEERAELNQSEEAEMPENGAGGP   393
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Figure 1 Isolation and structure of mouse esRAGE

(**A**) Schematic representation of the mouse RAGE gene and esRAGE mRNA. Open and shaded boxes indicate exons and introns respectively. Arrows indicate the positions of the primers used in RT–PCR: F1 and R1 in exons 1 and 11 respectively; F2 and R2 in exon 1–2 boundary and intron 9 respectively; F3 and R3 in exons 9 and 11 respectively. TM, transmembrane region. (**B**) Amplification of mouse esRAGE cDNA from brain polysomal poly(A)$^+$ RNA. Sizes are indicated in bp. (**C**) Alignment of the deduced amino acid sequences of the mouse esRAGE, mouse full-length membrane-bound RAGE and human esRAGE. Amino acid residues are numbered starting from the first methionine residue. The putative signal sequence and transmembrane region are underlined and boxed respectively. Asterisks indicate identical amino acids. The arrowhead indicates a proteolytic site for generation of sRAGE protein [11].

and 76777–76794 respectively of GenBank® accession number AF030001; F2 and R2 in Figure 1A) that corresponded to the boundary of exons 1 and 2, and intron 9 respectively (Figure 1A). The thermal cycling parameters were 95 °C for 30 s for denaturation, 63 °C for 1 min for annealing and 72 °C for 1 min for elongation. An aliquot (10 μl) of the RT–PCR product was electrophoresed on a 2 % agarose gel containing ethidium bromide, and amplified cDNAs were cloned into pCR2.1 (Invitrogen). Plasmid DNAs were purified with a Flexprep plasmid isolation kit (Amersham Biosciences), and their nucleotide sequences were determined with an ABI377 DNA sequencer (Applied Biosystems).

Stable transfection of COS-7 cells with expression vectors

The monkey-kidney-derived cell line, COS-7, was maintained in DMEM (Dulbecco's modified Eagle's medium), supplemented with 10 % (v/v) FBS (foetal bovine serum), 100 units/ml penicillin G and 100 μg/ml streptomycin. The newly cloned mouse esRAGE cDNA, the known mouse full-length RAGE cDNA that had been amplified with 5′-CACCATGCCAGCGGGGAC-3′ and 5′-GAGAATTCCATCACACAGGCTCGATC-3′ (nucleotides 2–19 and 1227–1245 of GenBank® accession number L33412 respectively; EcoRI site underlined) and mouse sRAGE cDNA that had been amplified with 5′-CACCATGCCAGCGGGGAC-3′ and 5′-GAGAATTCTTAACCTTCAGCTGGCCCCTC-3′ (nucleotides 1–18 and 977–994 of GenBank® accession number AB207883 respectively; EcoRI site underlined) to code for another secretory RAGE whose C-terminal end is PAEG (equivalent to a proteolytic form reported in [11]) were inserted into a pCI-neo mammalian expression vector (Promega) and sequence-verified. COS-7 cells (1×10^7 cells) were transfected with 20 μg each of the pCI-neo plasmids constructed by electroporation [8]. The human full-length RAGE and esRAGE expression pCI-neo vectors [8] were also used to transform the COS-7 cells. After incubation at 37 °C for 5 days, cell lysates were prepared as described previously [8]. To prepare conditioned media, confluent cultures of each transfectant were incubated for 24 h in serum-free media, which were then collected and centrifuged at 13000 ***g*** for 10 min, and the resultant supernatants were used as conditioned media.

RT–PCR

To determine tissue levels of esRAGE-encoding mRNA, total RNAs were isolated by the guanidinium thiocyanate method from various tissues of RAGE-null or wild-type mice, and were analysed by RT–PCR using the same primer set that was used for esRAGE cDNA isolation (F2 and R2 in Figure 1A, corresponding to exon 1–2 boundary and intron 9). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA levels were determined as internal control using the primer described previously [5]. The amount of RNA templates (100 ng) and number of amplification cycles (35 cycles for mouse esRAGE and 30 cycles for GAPDH) were chosen in semi-quantitative ranges where reactions proceeded linearly. Aliquots (10 μl) of each RT–PCR product were electrophoresed on 2 % agarose gels containing ethidium bromide. The ratio of esRAGE mRNA to full-length membrane-bound RAGE was determined by RT–PCR using polysomal poly(A)$^+$ RNA as a template and 5′-CGATGAGGGGCCAGCTGAAG-3′ and 5′-CCTGGCTTTCCGGGGCCTTC-3′ (nucleotides 974–993 and 1121–1140 respectively of GenBank® accession number L33412; F3 and R3 in Figure 1A) as primers common to the two splicing variants.

Antibody production

Mouse esRAGE-specific antibody against the unique C-terminal amino-acid peptide (PAEGEGLD) was raised in rabbits and affinity-purified with an antigen peptide-conjugated column according to the procedures as described previously [8].

SPR (surface plasmon resonance) assay

Conditioned medium from a serum-free culture of mouse esRAGE-expressing COS-7 cells was applied to a CM5 research grade sensorchip (BIAcore) to immobilize mouse esRAGE proteins. Coupling was achieved with an amine coupling kit (BIAcore) to a density of approx. 5000 RU (response units), according to standard amine coupling procedures. An equivalent volume of serum-free conditioned medium from non-transfected COS-7 cells was used as control. Binding between glyceraldehyde-derived AGE–BSA [8] and the immobilized esRAGE fraction was examined using a BIAcore 2000 system. The flow buffer used contained 10 mM Hepes, pH 7.4, 0.15 M NaCl, 3 mM sodium EDTA and 0.005 % (v/v) surfactant P-20. Association and dissociation were measured at 25 °C at a flow rate of 20 μl/min. The sensorchips were regenerated by washing with 10 mM NaOH and 0.5 % (w/v) SDS.

NF-κB (nuclear factor κB)–luciferase assay

C6 rat glioma cells were used for this assay according to procedures described previously [4]. Briefly, cells were transfected with a plasmid encoding luciferase cDNA under an enhancer element containing five NF-κB-binding sites (pNF-κB-Luc; Stratagene) and a plasmid containing full-length human RAGE cDNA. Stable transfectants were selected and cloned. The expression of RAGE was verified by Western blotting. A typical clone was used for subsequent analyses. After a 24 h pre-incubation in DMEM supplemented with 0.1 % FBS, the cells were stimulated for 4 h. Luciferase activity was determined using the Luciferase Assay system (Promega) and was measured in a luminometer (Fluoroscan Ascent FL; Labsystems).

Western blot analysis

Tissues from RAGE-null or wild-type mice were disrupted in lysis buffer [Ca^{2+}- and Mg^{2+}-free PBS, containing 1 % (v/v) Nonidet P-40, 0.5 % (w/v) deoxycholate, 10 mM EDTA, 0.1 % (w/v) SDS and 1 mM PMSF] using a Polytron tissue homogenizer as described previously [5]. Homogenates were centrifuged at 20000 ***g*** for 30 min, and supernatants were used for assays. Protein concentrations were determined using Coomassie Blue protein assay reagents (Pierce) using the method of Bradford [12]. Proteins were separated by SDS/PAGE (12.5 or 5–20 % gradient) and electroblotted on to PVDF membranes (Millipore). Membranes were blocked with 5 % (w/v) non-fat dried milk in PBS and 0.1 % (v/v) Tween 20, and then incubated with 0.5 μg/ml of immunoaffinity-purified mouse esRAGE-specific polyclonal antibody or a 1:500 dilution of anti-(RAGE extracellular domain) antibody [8,9]. After incubation with a 1:2000 dilution of horseradish-peroxidase-conjugated anti-rabbit IgG, the immune complexes were visualized with an ECL® (enhanced chemiluminescence) detection system (Amersham Biosciences).

RESULTS

Isolation of cDNA for mouse esRAGE

To see whether an esRAGE-like endogenous secretory isoform of RAGE occurs in mice, we attempted to isolate a cDNA coding for

it. For this purpose, polysomal poly(A)$^+$ RNA was isolated from mouse brain and was used as a template for RT–PCR cloning, because esRAGE expression was clearly marked in human cerebral neuronal cells [9]. Two sets of murine RAGE gene-specific primers were designed and synthesized, according to the organization of the human esRAGE-encoding mRNA [8] (Figure 1A). First, we amplified cDNAs with primers corresponding to exons 1 and 11 (Figure 1A). Next, we conducted a nested PCR with the first RT–PCR products as template and with the second set of primers corresponding to the exon 1–2 boundary and intron 9 (Figure 1A). As a result, the products gave a single band when electrophoresed on an agarose gel (Figure 1B). The DNA was cloned into a PCR2.1 plasmid and subjected to nucleotide sequencing. The sequence determined matched perfectly with the sequence of the exonic segments of the reported mouse RAGE gene (GenBank® accession number L33421), spanning exons 2–9, and that of intron 9. The largest open reading frame encoded a 334-amino-acid protein with an N-terminal putative signal sequence and with one V-region-like and two C-region-like extracellular immunoglobulin domains. The asparagine-linked glycosylation site resided at amino acid residues 25 and 80. The persistence of intron 9 resulted in a frame shift, giving rise to a unique C-terminal four-amino-acid sequence followed by an ochre stop codon UAA. Accordingly, the deduced protein was devoid of a hydrophobic transmembrane domain, which is contained in the full-length membrane-bound counterpart (Figure 1C). The protein encoded by this cDNA was thus considered as the mouse orthologue of esRAGE that was generated by alternative splicing of the RAGE gene transcript. The predicted amino acid sequence of mouse esRAGE is aligned with that of the human homologue in Figure 1(C). A comparison of the two sequences revealed 75.5 % identity (Figure 1C).

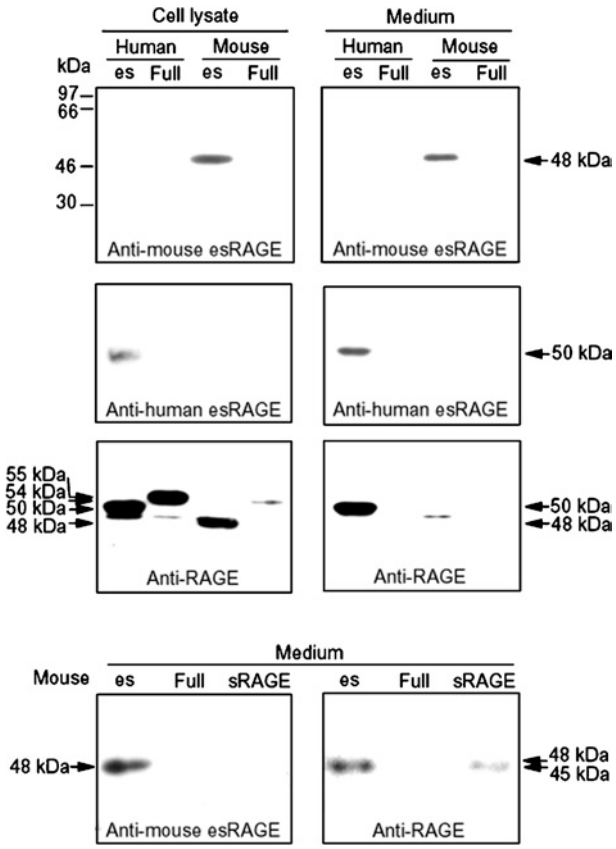

Figure 2 Recombinant expression of RAGE cDNAs in COS-7 cells

Cell lysates (10 μg) and conditioned media (30 μl) of the transfected COS-7 cells were analysed by Western blotting as described in the Experimental section. es, esRAGE cDNA-expressing-vector-transfected cells; Full, full-length RAGE cDNA-transfected cells; sRAGE, mouse sRAGE (equivalent to the proteolytically cleaved form [11])-encoding cDNA-transfected cells. The mouse-esRAGE-specific antibody recognized only mouse esRAGE at 48 kDa. The human-esRAGE-specific antibody recognized only human esRAGE at 50 kDa. A polyclonal anti-(RAGE extracellular domain) antibody (Anti-RAGE) [8,9] recognized all of these human and mouse RAGE variant proteins. Molecular-mass sizes are indicated in kDa.

Evaluation of the function of esRAGE as a decoy receptor

To check whether the putative esRAGE cDNA actually yields the soluble RAGE protein, we prepared an antibody against the unique C-terminus (PAEGEGLD) of putative mouse esRAGE protein. Then, COS-7 cells were transfected with a pCI-neo expression vector carrying the murine cDNA. Cell lysates and conditioned media of the transfectant were subsequently analysed by Western blotting. pCI-neo vectors carrying cDNA for mouse full-length RAGE, human full-length RAGE and human esRAGE were used as controls. Immunoblot analyses of both cell lysates and media from the putative esRAGE cDNA-transfected cells showed a single band at 48 kDa that reacted with the mouse esRAGE-specific antibody (Figure 2). The mouse esRAGE-specific antibody did not react with the 54 kDa mouse full-length RAGE protein, 45 kDa mouse sRAGE whose C-terminus was PAEG or the known human RAGE variants (Figure 2). On the other hand, an antibody against the extracellular domain of RAGE recognized the murine cDNA-derived 48 kDa secretory protein, the 45 kDa sRAGE and the human RAGE (Figure 2). The results thus indicate that the cloned mouse mRNA was translated as deduced, and that the protein product was secretory. In addition, it was concluded that the C-terminal EGLD sequence, which was present in 48 kDa esRAGE, but not in 45 kDa sRAGE, was essential for the recognition by the anti-(mouse esRAGE) antibody.

Next, whether the secreted protein had an ability to bind the AGE ligand was assessed by an SPR assay. For this, the conditioned medium from COS-7 cells which had been stably transfected with the cDNA expression vector was employed. The concentration of mouse esRAGE protein of this preparation was estimated to be 12 μg/ml, based on the data obtained by Western blotting with purified recombinant mouse sRAGE protein as a standard (Figure 3A). As shown in Figure 3(B), conditioned medium from COS-7 cells transformed by the cDNA-carrying vector gave a positive sensorgram with glyceraldehyde-derived AGE–BSA, the anti-(mouse esRAGE) antibody and the antibody against the extracellular domain of RAGE that recognized both the human and mouse proteins. Conditioned medium from mock-transformed COS-7 cells gave no signal (Figure 3B).

Next, we tested the effect of mouse esRAGE on AGE-induced activation of a transcriptional factor NF-κB, one of the most consistent cellular responses to AGE–RAGE interactions [4,13]. Full-length-RAGE-expressing C6 glioma cells were exposed to glyceraldehyde-derived AGE–BSA with or without treatment with conditioned medium from COS-7 cells transformed by the mouse esRAGE cDNA expression vector. AGE increased NF-κB-dependent luciferase activity, and this induction was significantly inhibited by sRAGE and by the conditioned medium containing mouse esRAGE, but not by control medium from mock-transformed COS-7 cells (Figure 4).

The results obtained thus indicated that the isolated cDNA actually coded for the murine orthologue of esRAGE.

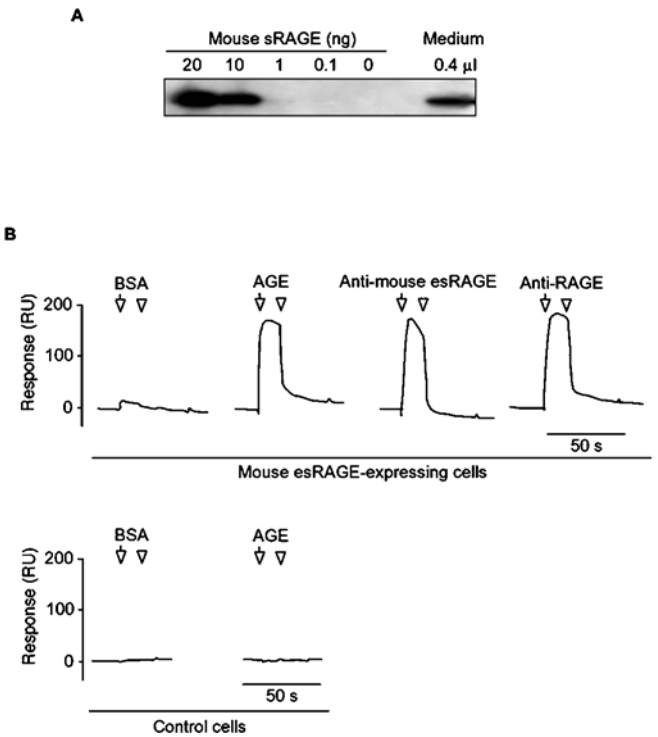

Figure 3 Binding of mouse esRAGE to AGE

(**A**) Estimation of a concentration of mouse esRAGE secreted into the culture medium by Western blotting using a polyclonal anti-(RAGE extracellular domain) antibody. Purified recombinant mouse sRAGE was used as a standard. The conditioned medium (0.4 μl) was equivalent to approx. 4.8 ng of sRAGE. (**B**) SPR assay was performed as described in the Experimental section. Non-glycated BSA (BSA) or glyceraldehyde-derived AGE–BSA (AGE) (150 μg/ml) [8] was passed over a CM5 sensorchip of BIAcore 2000, on which secreted mouse esRAGE protein had been immobilized. On a control chip, equivalent volumes of control non-transfected COS-7 cell media were immobilized. Immobilization of mouse esRAGE on a sensorchip was confirmed by a positive response when 10 μg/ml anti-(mouse esRAGE) antibody or an anti-(RAGE extracellular domain) antibody was injected. Positive response was detected on injection of AGE, but not of control BSA. The white arrow indicates the injection point. At 15 s after the injection (white arrowhead), the mobile phase was changed back to the buffer alone.

esRAGE mRNA expression in mouse tissues

Total RNAs were isolated from various tissues of C57BL/6J mice and were analysed by RT–PCR with primers corresponding to the exon 1–2 boundary and intron 9. As shown in Figure 5(A), the esRAGE mRNA-derived 1004 bp band was clearly present in the brain, lung, kidney and small intestine. RNAs from the same tissues of the RAGE-null mice gave no band. The esRAGE mRNA level was the highest in the lung.

To determine the relative abundance of mRNAs for full-length RAGE and esRAGE in the tissues where esRAGE mRNA was detected, polysomal poly(A)$^+$ RNAs from wild-type mice were analysed by RT–PCR with primers corresponding to exons 9 and 11 that were contained in both full-length and secretory-form-encoding variants. Three bands were detected in this analysis (Figure 5B). The 161 bp band was sequence-verified to represent exons 9 + 10 + 11 from the full-length RAGE mRNA; the 278 bp and 364 bp bands represented exon 9 + intron 9 + exons 10 + 11 and exon 9 + intron 9 + exon 10 + intron 10 + exon 11 respectively, and were derived from the mRNA for esRAGE. From the band intensity, the ratio of the esRAGE mRNA to the full-length RAGE mRNA was estimated to be approx. 1:1.8, 1:0.9, 1:1.6 and 1:0 in the brain, lung, kidney and small intestine respectively.

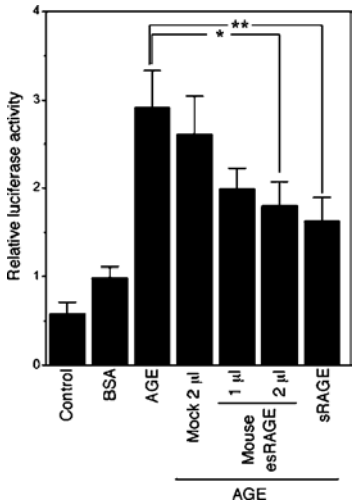

Figure 4 Inhibitory effect of mouse esRAGE on AGE-induced NF-κB activation

C6 glioma cells were stably transfected with an NF-κB-responsive *cis*-reporter gene construct together with a full-length RAGE expression vector as described in the Experimental section. The cells were stimulated by no addition (control), or with 50 μg/ml BSA (BSA), 50 μg/ml glyceraldehyde-derived AGE–BSA alone (AGE), 50 μg/ml AGE–BSA plus additives: 1 or 2 μl of concentrated conditioned media from mock-transfected cells (Mock 2 μl) or mouse esRAGE cDNA-transfected cells (Mouse esRAGE 1 μl and 2 μl, corresponding to $\sim$0.9 and $\sim$1.8 μg of esRAGE proteins respectively), or 20 μg of sRAGE (sRAGE) (kindly provided by Mitsubishi Pharma Corporation). Relative luciferase activity is shown. Results are means $\pm$ S.E.M. ($n = 3$). $^*P < 0.04$, $^{**}P < 0.02$; statistical analysis was performed by ANOVA combined with a multiple comparison test (Scheffe's type).

Detection of esRAGE proteins in mouse tissues

To prove that esRAGE proteins were produced in mouse tissues, we conducted Western blot and immunohistochemical analyses using the anti-(mouse esRAGE) antibody. Figure 5(C) shows immunoreactive bands at 48 kDa in the brain, lung, kidney and small intestine from wild-type mice, but not from RAGE-null mice (Figure 5C). In kidney, an additional band was detected at 43 kDa. The rank order of relative abundance of esRAGE protein was small intestine > brain > kidney > lung.

Next, sections of mouse brain, lung, kidney and small intestine were stained for esRAGE. Positive-signals were present in neurons of the cerebrum, bronchial epithelium, endothelial cells, tubular cells of kidney and epithelial cells of small intestine from wild-type mice (Supplementary Figure S1 at http://www.BiochemJ.org/bj/396/bj3960109add.htm). No signal was detected in non-diabetic or diabetic RAGE-null mice (Supplementary Figure S1). Expressions of esRAGE protein was increased in the kidney, but not in the other tissues, of the diabetic mice (Supplementary Figure S1). Western blotting and RT–PCR analyses confirmed the increase in esRAGE protein and its mRNA in diabetic kidney (Supplementary Figure S2 at http://www.BiochemJ.org/bj/396/bj3960109add.htm).

DISCUSSION

In the present study, we have identified for the first time the mouse orthologue of esRAGE. The mRNAs for mouse esRAGE were generated by alternative splicing and consisted of two forms: one that had the intron 9 sequence and the other that had introns 9 and 10 (Figures 1A and 5B). Persistence of the intron 9 sequence resulted in frame shifting with a stop codon UAA; therefore the two mRNA forms should yield one and the same

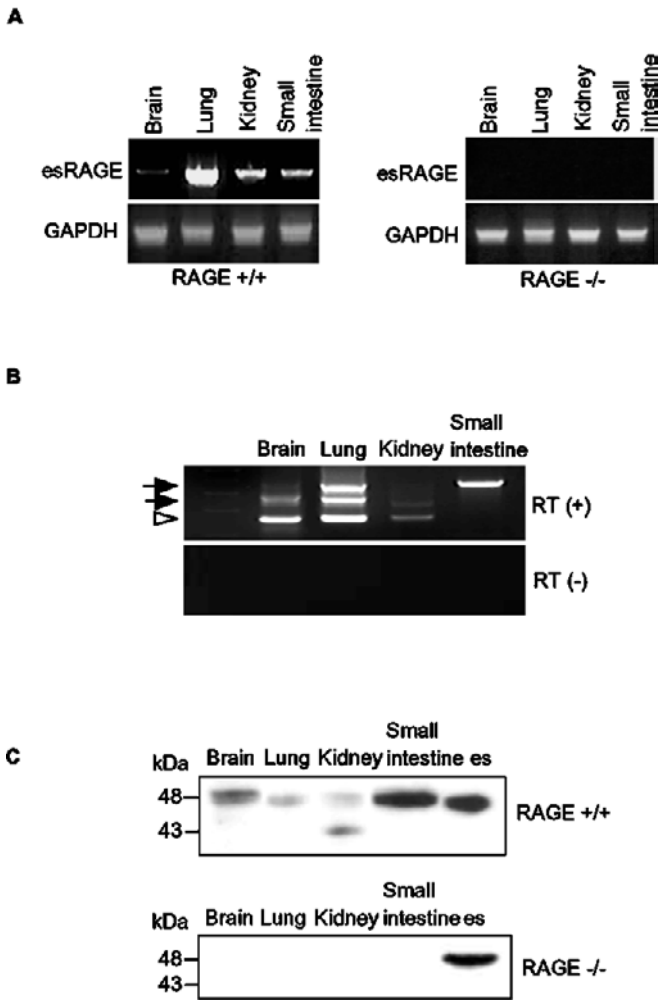

Figure 5 Detection of esRAGE mRNA and protein in mouse tissues

(**A**) Total RNA (100 ng) from brain, lung, kidney and small intestine of wild-type (+/+) or RAGE-null (−/−) mice (C57BL/6J) at 16 weeks of age was analysed by RT–PCR as described in the Experimental section. (**B**) Determination of relative abundance of esRAGE and full-length RAGE mRNAs. Polysomal poly(A)$^+$ RNAs were analysed with primers common to both esRAGE and full-length RAGE mRNA as described in the Experimental section. All bands were sequence-verified: the 161 bp band (open arrowhead) represents the full-length RAGE mRNA, while the 278 and 364 bp bands (black arrows) represent the esRAGE mRNA. (**C**) Immunoblotting for esRAGE protein in C57BL/6J mouse tissues. Proteins were extracted from the brain, lung, kidney and small intestine of wild-type (RAGE+/+) or RAGE-null (RAGE−/−) mice at 16 weeks of age, and were analysed by Western blotting using a polyclonal anti-(mouse esRAGE) antibody. The amounts of protein loaded for the Western blotting were: brain, 8 μg; lung, 15 μg; kidney, 10 μg; small intestine, 5 μg. Molecular-mass sizes are indicated in kDa.

protein product. The deduced protein had 334 amino acids with a signal sequence and a unique four-amino-acid C-terminal stretch, but lacked the transmembrane domain (Figure 1C). The human mRNA variant for esRAGE isolated from primary cultured vascular endothelial cells was exclusively devoid of the exon 10 sequence [8]; this suggested that there may be species variation in splicing events to generate esRAGE mRNAs. Transfection experiments demonstrated that the mouse esRAGE mRNAs yielded 48 kDa esRAGE, and that the proteins were secreted into the medium (Figure 2). Conditioned media from the murine esRAGE cDNA-expressing transfectant exhibited AGE-binding ability and inhibition of AGE-induced NF-κB activation (Figures 3 and 4).

The physiological occurrence of esRAGE in mouse tissues was also demonstrated in the present study. The mRNA for esRAGE (Figure 5A and 5B) and its protein product (Figure 5C, and Supplementary Figures S1 and S2) were detected in the brain, lung, kidney and small intestine. This suggests that the rank orders for mRNA and protein abundance were not in agreement; thus for mRNA it was lung > kidney > small intestine > brain, but, for protein, it was small intestine > brain > kidney > lung. Efficiency of translation of the mRNA, post-translational processing of the protein, deposition after secretion in the tissue and other factors that can influence the tissue distribution of esRAGE may vary among these tissues. The size of the major immunoreactive species was consistently 48 kDa in those tissues (Figure 5C), as was that expressed in COS-7 cells (Figure 2). It seems unlikely that the 43 kDa kidney protein (Figure 5C) was due to alternative splicing, because kidney RNA yielded only a single band when analysed by RT–PCR with primers corresponding to exon 1–2 boundary and intron 9 (Figure 5A and Supplementary Figure S2). The 43 kDa protein may thus be due to alternative glycosylation or proteolysis of esRAGE. The patterns of immunostaining for mouse esRAGE in the brain, lung, kidney and small intestine (Supplementary Figure S1) were similar to those seen in human subjects [9].

Recently, a variety of RAGE ligands have been identified, including not only AGE, but also amphoterin/HMGB1 [14], S100/calgranulins [3], Alzheimer amyloid β-proteins [15] and Mac-1 [16]. A part of those ligands should have evolved to regulate certain physiological processes, such as pro-inflammatory reactions. It also seems to be unlikely that esRAGE (multiligand binding) has evolved to only capture ('non-physiological') glycated ligands. Nevertheless, as a result of increases in human lifespan, some ligands might 'abuse' the cell-surface signalling RAGE under certain pathological conditions, such as diabetic complications, atherosclerosis and neurodegenerative disorders. It has been reported that the sRAGE produced artificially using recombinant gene technology can bind ligands and prevent progression of diabetic vasculopathy and atherosclerosis in experimental animals [6]. In the present study, expression of esRAGE protein in diabetic wild-type mice was observed to be increased in kidney when compared with the non-diabetic wild-type mice (Supplementary Figure S1). This was confirmed by RT–PCR and immunoblot analyses of RNA and proteins isolated from diabetic and non-diabetic animals (Supplementary Figure S2). The production and up-regulation of esRAGE would have physiological significance, since esRAGE in tissues should act as a decoy receptor and protect cells and tissues from ligand-dependent injury. Recently, we found that diabetic patients with high serum esRAGE levels were more resistant to developing early retinal complications than those with low esRAGE levels [17]. Moreover, plasma esRAGE is found to be a novel biomarker and a potential protective factor in metabolic syndrome and atherosclerosis [18]. These data suggest that esRAGE will confer protection against diabetic complications and atherosclerosis. Hanford et al. [11] recently reported the existence of a soluble form of RAGE which would be produced by C-terminal truncation in mouse lung tissue and not by alternative splicing. Based on its purported size and structure, we speculate that it is possible that this form can be generated not only from the full-length membrane-bound form, but also from murine esRAGE by further proteolysis, and that it may also work as a decoy for RAGE ligands in some physiological or disease conditions.

In conclusion, the present study has identified esRAGE in mice, providing a basis for investigating the roles of this multiligand decoy receptor in healthy and diseased animal models.

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