The receptor for advanced glycation end-products (RAGE) directly binds to ERK by a D-domain-like docking site

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Abstract The receptor for advanced glycation end-products (RAGE)-mediated cellular activation through the mitogen-activated protein kinase (MAPK) cascade, activation of NF-κB and Rho family small G-proteins, cdc42/Rac, is implicated in the pathogenesis of inflammatory disorders and tumor growth/metastasis. However, the precise molecular mechanisms for the initiation of cell signaling by RAGE remain to be elucidated. In this study, proteins which directly bind to the cytoplasmic C-terminus of RAGE were purified from rat lung extracts using an affinity chromatography technique and identified to be extracellular signal-regulated protein kinase-1 and -2 (ERK-1/2). Their interactions were confirmed by immunoprecipitation of ERK-1/2 from RAGE-expressing HT1080 cell extracts with anti-RAGE antibody. Furthermore, the augmentation of kinase activity of RAGE-bound ERK upon the stimulation of cells with amphoterin was demonstrated by determining the phosphorylation level of myelin basic protein, an ERK substrate. In vitro binding studies using a series of C-terminal deletion mutants of human RAGE revealed the importance of the membrane-proximal cytoplasmic region of RAGE for the direct ERK–RAGE interaction. This region contained a sequence similar to the D-domain, a ERK docking site which is conserved in some ERK substrates including MAPK-interacting kinase-1/2, mitogen- and stress-activated protein kinase-1, and ribosomal S6 kinase. These data suggest that ERK may play a role in RAGE signaling through direct interaction with RAGE.

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Key words: Receptor for advanced glycation end-products; Extracellular signal-regulated kinase; Amphoterin

1. Introduction

The receptor for advanced glycation end-products (RAGE) is a member of the immunoglobulin superfamily of cell surface molecules [1]. It is well known as a multi-ligand receptor which interacts with various ligands including EN-RAGE/S100A12 which is a member of the S100/calgranulin family [2], amyloid-β [3,4], amphoterin [5] and advanced glycation end-products (AGEs) [6].

RAGE is expressed in vascular smooth muscle cells, endothelial cells, mononuclear phagocytes, mesangial cells, neurons, and lung alveolar epithelial cells [7,8]. Engagement of the ligands to RAGE induces expression of the receptor itself and amplifies the pro-inflammatory response leading to sustained cellular activation and tissue dysfunction by the receptor-dependent mechanism [9]. Although RAGE may not be essential to the initial pathogenesis of disorders, the distribution patterns of RAGE and its ligands reflect the involvement of RAGE in the amplification or progression of various diseases, such as diabetes [10,11], Alzheimer's disease [3,4], inflammation [2,9] and tumors [12].

The S100/calgranulins are a group of closely related proteins with two EF-hand domains and have a central role as a mediator in inflammatory disorders [13–15]. Hofmann et al. [2] have reported that RAGE–S100/calgranulin interaction participates in immune/inflammatory responses.

In Alzheimer's disease, deposition of amyloid-β-containing plaques in the brain correlates with progressive neuronal dysfunction leading to dementia. Neuronal RAGE has been shown to interact with amyloid-β resulting in cellular oxidant stress and triggering a pro-inflammatory pathway leading to activation of microglial cells [3,4].

AGEs resulting from non-enzymatic glycation of proteins and lipids gradually accumulate during normal aging and at an accelerated rate in diabetes. Hyperglycemia-driven accumulation of AGEs has been suggested to be involved in the pathogenesis of diabetic vascular disease. The interaction of AGEs with RAGE results in perturbation of a variety of vascular homeostatic functions and has been shown to play a major role in the development of diabetic vasculopathy [6,11].

Amphoterin, also called high mobility group 1, is a member of the family of high mobility group non-histone chromosomal DNA binding proteins [16–19]. When amphoterin is released from cells, its engagement with RAGE promotes neurite outgrowth of neuronal cells in the nervous system [20,21] as well as tumor growth and metastasis [12]. Srikrishna et al. have reported that interaction between amphoterin and RAGE depends on the carboxylated *N*-glycans of RAGE [22]. Interestingly, they showed that the glycans on RAGE have an important role in RAGE-dependent neurite outgrowth of N18 neuroblastoma cells.

Thus, RAGE interacts with a range of ligands leading to diverse cellular responses varying from cytokine secretion and increased cellular oxidant stress to cell survival, differentia-

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Abbreviations: ERK, extracellular signal-regulated kinase; AGE, advanced glycation end-product; RAGE, receptor for AGE; MAPK, mitogen-activated protein kinase; RSK, ribosomal S6 kinase; MnK, MAPK-interacting kinase; MSK, mitogen- and stress-activated protein kinase; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis

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tion, and proliferation. These responses are dependent on RAGE-mediated signals and gene expression. It has been shown that RAGE ligation by its ligands results in the activation of multiple signaling pathways, including the mitogen-activated protein kinase (MAPK) cascade [23], cdc42/Rac [20], and activation of NF-κB and Sp1 [20,23–25]. Several gene targets which are upregulated upon the activation of RAGE signaling pathways have been identified, including interleukin-6 [26], vascular endothelial cell growth factor [24,27], vascular cell adhesion molecule-1 [28] in endothelial cells, macrophage/colony-stimulating factor [4], tissue factor [29] in macrophage, and Bcl-2 [30] in neuronal cells. However, the molecular basis leading to the activation of these signaling pathways after RAGE/ligand ligation remains to be elucidated. It was reported that receptors with a highly charged cytosolic tail are critical for RAGE-mediated cellular activation because the cytosolic C-terminally truncated RAGE serves as a dominant negative receptor for blocking the effects of RAGE-dependent cellular activation [9,12,20]. These observations evidently indicate the existence of a signaling molecule(s) which associates with the cytoplasmic domain of RAGE.

In the present study, we aimed to identify the downstream effector(s), which can interact with the cytoplasmic tail of RAGE, using affinity purification techniques. Rat lung was used as a starting material for the purification of RAGE binding proteins, because the RAGE protein is expressed mainly in the lung tissue. Finally, we identified extracellular signal-regulated kinase-1 and -2 (ERK-1/2) as RAGE binding proteins and demonstrated their interactions in the cells. Our results may contribute to the understanding of the molecular basis of RAGE signaling.

2. Materials and methods

2.1. Cell culture and transfection

Human fibrosarcoma HT-1080 and mouse melanoma B16-F10 cells were maintained in medium A (RPMI 1640 medium supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, and 10% v/v fetal calf serum) at 37°C in a humidified 5% CO_2–95% air incubator. The cDNA fragment encoding the full-length human RAGE (NCBI accession number: 26787960) was amplified with 5′-TTGAATTCAGGATGGCAGCCGGAACAGCAG-3′ and 5′-TTCTCGAGTCAAGGCCCTCCAGTACTAC-3′. The amplified cDNA was digested with *Eco*RI and *Xho*I, and inserted into a mammalian expression vector, pcDNA3.1+ (Invitrogen, Carlsbad, CA, USA) that had been digested with the same restriction enzymes. HT1080 cells were transfected with the resultant plasmid, pcDNA-hRAGE using LipofectAMINE 2000 according to the manufacturer's instructions (Invitrogen). HT1080-hRAGE cells stably expressing human RAGE were selected and maintained in medium A supplemented with 0.5 mg/ml G418 (Life Technologies, Japan).

2.2. Recombinant proteins and antibodies

Fusion proteins with glutathione *S*-transferase (GST) or a hexahistidine tag were expressed by employing pGEX-6P1 (Amersham-Pharmacia) and pP_{RO}EX-HTa (Invitrogen) in the BL21(DE3) strain of *Escherichia coli* (Novagen, Madison, WI, USA).

Recombinant CR43-GST fusion protein was prepared and purified as follows. The cDNA fragment encoding the cytoplasmic C-terminal domain (aa 362–404) of human RAGE was subcloned into the *Bam*HI-*Xho*I site downstream of the sequence for GST in pGEX-6P1, yielding pGEX-CR43. This plasmid was transformed into the BL21(DE3) strain of *E. coli* and induced with isopropyl-β-D-thiogalactopyranoside to produce GST fusion proteins. The bacteria cells were suspended in phosphate-buffered saline (PBS) and vigorously sonicated followed by centrifugation at 10 000×*g* for 30 min. The resulting supernatants were applied on a glutathione beads column and then eluted with buffer E (50 mM Tris–HCl, pH 7.6, 120 mM NaCl, 10 mM glutathione). Purified GST fusion proteins were dialyzed against PBS.

For preparing two C-terminal deletion mutants of CR43-GST (CR34-GST and CR18-GST fusion proteins), nucleotides corresponding to Gln^{379} and Glu^{395} of human RAGE in plasmid pGEX-CR43 were point-mutated into the stop codon using oligonucleotides: CR34-sense, 5′-CAGTCGGAGGAACCTTAGGCAGGCGAGAGTAG-3′; CR34-antisense, 5′-CTACTCTCGCCTGCCTAAGGTTCCTCCGACTG-3′; CR18-sense, 5′-GAAGGCCCCAGAAAACTAGGAGGAAGAGGAGG-3′; CR18-antisense, 5′-CCTCCTCTTCCTCCTAGTTTTCTGGGGCCTTC-3′ (QuikChange™ site-directed mutagenesis kit, Stratagene, La Jolla, CA, USA), yielding pGEX-CR34 and pGEX-CR18 respectively. Recombinant CR34-GST and CR18-GST proteins were prepared and purified as described above.

C-terminally truncated recombinant human amphoterin was expressed as a fusion protein with a hexahistidine tag to its C-terminus and purified from *E. coli* BL2(DE3) (Novagen) harboring the pP_{RO}EX-hAmphoterinΔC plasmid using a Ni-NTA agarose column (Qiagen, Japan). pP_{RO}EX-hAmphoterinΔC was constructed by inserting the cDNA encoding human amphoterin (1–185 aa) into pP_{RO}EX-HTa. Specific binding of the C-terminally truncated amphoterin with RAGE was confirmed by a binding assay.

The monoclonal antibody against RAGE was prepared as follows. Recombinant human soluble RAGE protein (319 aa) was purified from the culture supernatant of baculovirus-infected ExpreSF+™ cells with the FPLC system (Amersham-Pharmacia) using HiTrap™ SP and HiTrap™ Butyl columns. WKY/NCrj rats were immunized with the recombinant RAGE protein. Lymph node cells were harvested from the immunized rats and fused with mouse myeloma cells SP2/O-Ag14 (Dainippon Seiyaku, Japan). One of the established monoclonal antibodies was named 11F2. The RAGE polyclonal antibody raised against a peptide corresponding to the N-terminal immunoglobulin variable region-like domain of human RAGE was kindly provided by Metabolism and Cardiovascular Diseases, Novartis Pharmaceuticals Corp. (East Hanover, NJ, USA). These antibodies were affinity-purified from the ascites fluids of nude mice or the sera of rabbits.

Rabbit anti-ERK (K-23, #sc-94) and anti-active® MAPK polyclonal antibodies (#V8031) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Promega (Madison, WI, USA), respectively.

2.3. Affinity purification and amino acid sequencing of RAGE binding protein

Lung tissues obtained from 10 male Wistar rats were homogenized with T-PER Tissue Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA) containing a cocktail of protease inhibitors, Complete™ (Roche, Mannheim, Germany), and the homogenate was centrifuged at 100 000×*g* for 1 h at 4°C. The supernatant (150 ml) was recovered and used as a lung extract. Glutathione-Sepharose 4B (Amersham Pharmacia) saturated with the recombinant CR43-GST fusion protein or control GST was used to affinity-purify the CR43 binding proteins. The rat lung extract (total protein: 1050 mg) was pre-cleared with GST-glutathione beads (bead volume: 150 μl) followed by incubation with 50 μl of CR43-GST-glutathione beads by gently rotating for 2 h at 4°C. The beads were washed five times with 10 ml of ice-cold extraction buffer diluted with an equal volume of PBS, and further washed twice with PBS. Then, proteins were eluted with buffer E. After the eluate was dialyzed against PBS and concentrated, the protein solution containing p39 and p43 was subjected to two-dimensional polyacrylamide gel electrophoresis (2D-PAGE: isoelectric focusing GE and SDS–PAGE) in the denaturing conditions. The bands of p39 and p43 stained with Coomassie brilliant blue R250 were cut out and washed extensively with MilliQ water. Each piece of the gel blocks was sent to APRO Science Inc. (Tokushima, Japan) for their internal amino acid sequencing. In brief, p39 and p43 were digested with trypsin. The resulting peptides were separated onto a column of TSK gel ODS-80Ts QA with a 0–90% gradient of acetonitrile in 0.1% trifluoroacetic acid and then the peptide sequences determined with the Procise 494 HT protein sequencing system (Applied Biosystems Japan).

2.4. Immunoprecipitation, Western blotting and in vitro kinase assay

HT1080-hRAGE cells were grown in medium A until 80% confluence. Cells (1.5×10^7) were washed with serum-free RPMI 1640 medium supplemented with 1% bovine serum albumin (Sigma, St. Louis,

MO, USA) and starved in the same medium for 8 h. Then, amphoterin (25 μg/ml) was added to the cell culture and incubated for a certain period. HT1080-hRAGE cells were lysed with a Triton X-100 solubilization buffer composed of 20 mM Tris–HCl (pH 7.6), 120 mM NaCl, 1 mM EDTA, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 1 mM sodium orthovanadate, 1% Triton X-100 containing Complete™ on ice. The lysate was preincubated with protein A-Sepharose (Amersham Pharmacia) for 1 h at 4°C and then incubated with a rabbit anti-RAGE IgG coupled to protein A-Sepharose. After 2 h incubation at 4°C, the beads were washed four times with a Triton X-100 solubilization buffer. The immunoprecipitates were then used for further analyses, such as Western blotting probed with anti-ERK antibody and in vitro kinase assay. In brief, for the analysis of ERK binding to RAGE, the immunocomplexes were separated in SDS–PAGE gels and electrotransferred onto polyvinylidene difluoride filters. Filters were blocked with 5% non-fat dry milk powder and incubated with an anti-ERK polyclonal antibody, K-23 (#sc-94, Santa Cruz) followed by incubation with a peroxidase-conjugated anti-rabbit IgG antibody (NA9340V, Amersham Biosciences), and then the bands of ERK proteins were visualized with enhanced chemiluminescence (Amersham Biosciences). ERK kinase activities contained in the RAGE immunoprecipitates were measured in vitro using a MAPK assay kit (Upstate, Lake Placid, NY, USA). The immunoprecipitates (5 μl of bead volume) were further washed twice with 75 μl of 1×assay dilution buffer (ADB) and supplemented with 10 μl of each reagent, 1×ADB, kinase inhibitor cocktail (protein kinase (PK) C, PKA inhibitor peptides and compound R24571), MAPK substrate cocktail (myelin basic protein, MBP), and Mg/ATP cocktail. After incubation at 30°C for 20 min in a shaking incubator, a 2.5 μl aliquot of the reaction mixture was separated by SDS–PAGE and subjected to Western blotting, in which phosphorylation of MBP was detected with anti-phospho MBP antibody (Upstate).

2.5. In vitro binding experiments

Each of the extracts which were prepared from rat lung tissues, HT1080 and B16 cells were incubated with 25 μg of CR43 on glutathione-Sepharose beads (bead volume: 5 μl) for 2 h at 4°C. The beads were washed three times with wash buffer composed of 10 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 0.5% v/v Triton X-100, and then SDS sample buffer was added. The resultant eluate was subjected to SDS–PAGE followed by Western blotting as described before.

In the experiments using the recombinant proteins, phosphorylated and non-phosphorylated ERK-2 (#SE-137 and #SE-139, Biomol Research Laboratories, Butler Pike, PA, USA), 25 μg of CR43 on glutathione-Sepharose beads (5 μl) was added into 300 μl of binding buffer (10 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% w/v bovine serum albumin, 0.5% v/v Triton X-100) containing 250 ng of phosphorylated ERK or non-phosphorylated ERK and incubated for 2 h at 4°C, and then the beads were washed and the bound proteins were eluted as described above. One tenth of each eluate was used for Western blotting analysis.

3. Results

3.1. Identification and purification of RAGE-associated proteins

To identify the molecules that specifically bind to CR43, the C-terminal cytoplasmic domain of RAGE, the rat lung extract was incubated with CR43-GST affinity beads. The proteins bound to the beads were co-eluted with CR43-GST by adding glutathione. Finally, two proteins with molecular masses of approximately 43 kDa (p43) and 39 kDa (p39) were detected in the glutathione eluate from a CR43-GST column but not from a control GST column (Fig. 1A); therefore, they were further purified by 2D-PAGE and subjected to amino acid sequencing. Database searching of the determined sequences (GTAGVVPVVPGE from p43, IEVEQALAHPYL from p39) revealed that p43 and p39 were ERK-1 and -2, respectively. The specific interaction of ERK with CR43-GST was confirmed by Western blotting using an antibody specific to phosphorylated ERK-1/2 (Fig. 1B), whereas there was no interaction of CR43-GST with c-Jun N-terminal kinase (JNK) and p38 MAPK (data not shown). It was also demonstrated that ERK-1/2 from human fibrocarcinoma HT-1080 and mouse melanoma B16-F10 cells as well as ERK-1/2 from the rat lung extract bound to CR43-GST protein.

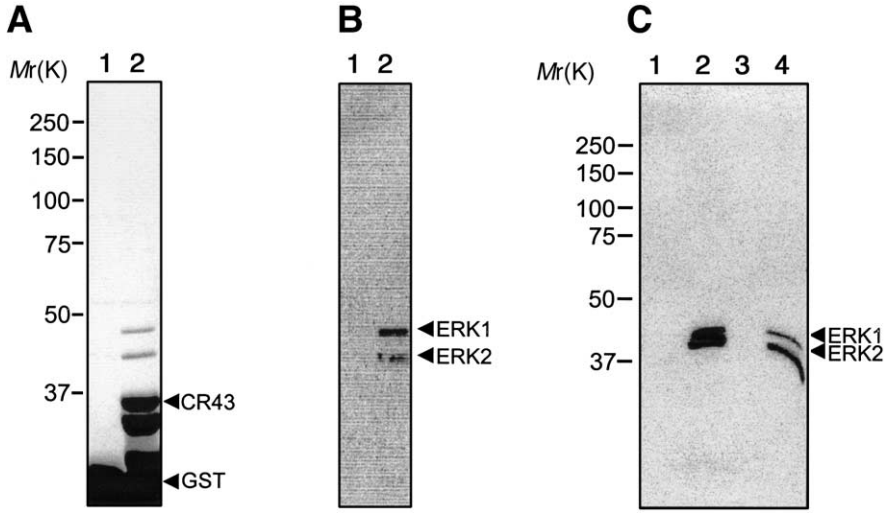

Fig. 1. Identification of p43 (ERK-1) and p39 (ERK-2) as RAGE binding proteins. A: Affinity purification of p43 and p39. The rat lung extract which was prepared as described in Section 2 was subjected to affinity binding to GST beads (lane 1) or human CR43-GST (lane 2) beads, and the bound proteins were eluted by the addition of buffer E containing glutathione. The eluates were subjected to SDS–PAGE, followed by Coomassie brilliant blue staining. B: Identification of the RAGE binding proteins. The p39 and p43 proteins were recovered from the pieces of gel blocks, digested with trypsin and their N-terminal amino acid sequences determined. The same eluates as shown in A were also probed in a Western blot with the antibody specific for phosphorylated ERKs (anti-active MAPK). Lane 1, GST; lane 2, CR43-GST. C: HT1080 and B16-F10 cells were extracted with 1% Triton X-100 and probed with anti-active MAPK. In vitro binding experiments were carried out using cell extracts. Lane 1, GST (HT1080 cells); lane 2, CR43-GST (HT1080 cells); lane 3, GST (B16 cells); lane 4, CR43-GST (B16 cells).

Next, we examined whether their interaction is direct or mediated by any adapter protein which can bind both RAGE and ERK. As shown in Fig. 2, both phosphorylated and non-phosphorylated forms of the recombinant ERK-2 protein interacted with CR43-GST protein, indicating that CR43-GST directly binds with ERK. In our experimental conditions, their interaction appeared to be independent of the phosphorylation of ERK. Furthermore, we obtained the same results when a fusion protein (mouse CR43-GST) of the cytoplasmic domain of mouse RAGE with GST was used instead of human CR43-GST for the in vitro binding assay, suggesting a conserved ERK binding ability of RAGE between species.

3.2. Association of ERK with RAGE in cells

To examine the interactions between RAGE and ERK in cells, HT1080-hRAGE cells were solubilized with 1% Triton X-100 and immunoprecipitated with an anti-RAGE polyclonal antibody. The resulting immunoprecipitates were probed with an anti-ERK antibody. As shown in Fig. 3A, ERK was co-immunoprecipitated with an anti-RAGE polyclonal antibody after the engagement of amphoterin to RAGE, indicating that the ligand engagement can enhance the RAGE–ERK interaction in cells expressing RAGE. A maximal signal of ERK–RAGE interaction was observed at 20 min after exposure of the cells to amphoterin.

In order to demonstrate the kinase activity of ERK associated with RAGE, RAGE immunoprecipitates were prepared from parental HT1080 as well as HT1080-hRAGE cells with or without amphoterin stimulation. MBP was used as a substrate for phosphorylation by ERK in the presence of some inhibitors for other kinases such as PKA and PKC. As shown in Fig. 3B, higher activities of RAGE-bound MAPK were detected in the RAGE immunoprecipitates from HT1080-hRAGE cells (lanes 4–7) compared with those from control HT1080 cells (lanes 1–3). These kinase activities were detected

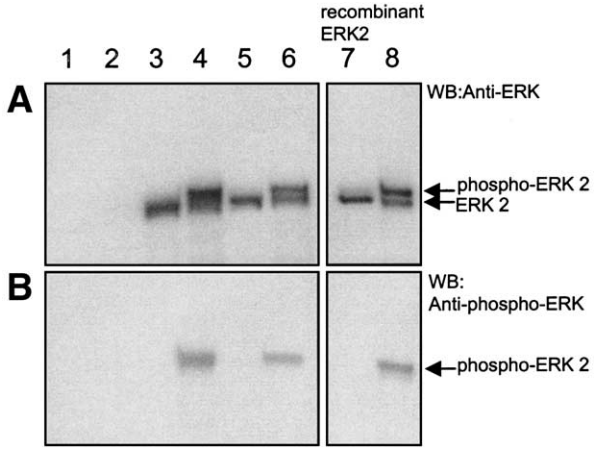

Fig. 2. Both phosphorylated and non-phosphorylated ERK-2 directly interact with CR43-GST protein. Phosphorylated (lanes 2, 4, and 6) and non-phosphorylated (lanes 1, 3, and 5) recombinant ERK-2 were mixed with GST (lanes 1 and 2), human CR43-GST (lanes 3 and 4), or mouse CR43-GST (lanes 5 and 6) proteins bound to glutathione-Sepharose beads and incubated for 2 h at 4°C. Their eluates and recombinant ERK proteins (lanes 7 and 8) were boiled with SDS sample buffer and separated by SDS–PAGE, followed by Western blotting (WB) using (A) anti-ERK antibody, K-23 or (B) anti-active phosphorylated MAPK polyclonal antibodies.

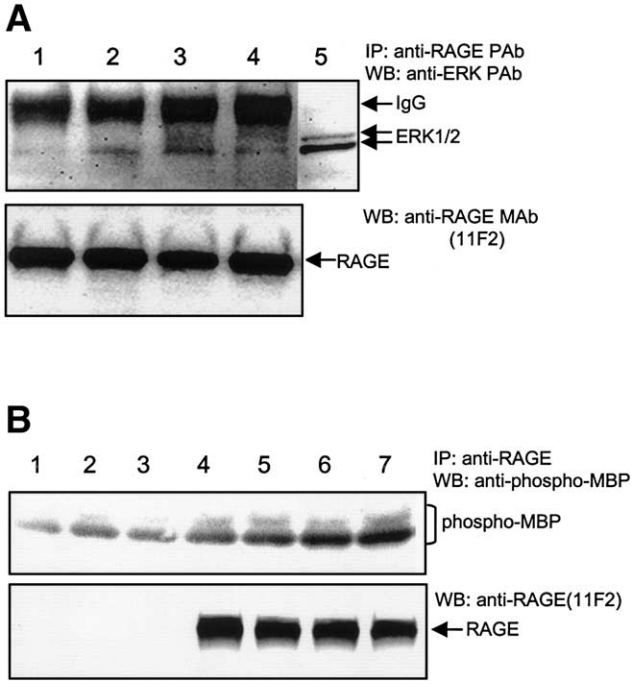

Fig. 3. ERK interacts with RAGE in HT1080 cells. A: RAGE–ERK interaction in HT1080 cells stably transfected with human RAGE cDNA. The cells (HT1080-hRAGE) were untreated (lane 1) or treated with amphoterin (25 μg/ml) for 5, 20, or 80 min (lanes 2–4, respectively) and then solubilized with Triton X-100, and the RAGE was immunoprecipitated (IP) with a rabbit anti-RAGE polyclonal antibody. The resulting immunoprecipitates were then analyzed by Western blotting (WB) using anti-ERK (K-23) or anti-RAGE monoclonal (11F2) antibodies. B: Kinase activities in RAGE immunoprecipitates. The RAGE immunoprecipitates were prepared from parental HT1080 (lanes 1–3) and HT1080-hRAGE cell lysates (lanes 4–7) in the same experimental conditions as in A. The cells were untreated (lane 4) or treated with amphoterin for 5, 20, or 80 min (lanes 1–3 and 5–7) prior to solubilization. Then, they were subjected to in vitro phosphorylation assay with MBP as an ERK substrate. The phosphorylation of MBP was detected by probing with anti-phospho-MBP antibody (clone P12, #05-429, Upstate) according to the manufacturer's instructions.

at 20 min after cell exposure to amphoterin and remained high for up to 80 min. Thus, the results indicate that amphoterin binding to the cell surface induced intracellular ERK–RAGE interactions and enhanced ERK kinase activity.

3.3. Domain involved in ERK and RAGE interaction

We next examined the effect of the C-terminal deletion of RAGE on the binding of ERKs by in vitro binding assay (Fig. 4A). The results indicated that the 18 amino acids in the juxtamembrane region (CR18) are sufficient for binding ERK (Fig. 4B). We also found that the membrane-proximal regions corresponding to amino acid residues 362–374 of RAGEs contain a sequence motif similar to the ERK docking sites of MAPK-interacting kinase 1/2 (MnK1/2), mitogen- and stress-activated protein kinase 1 (MSK1), and ribosomal S6 kinases pp90^{rsk} (RSKs) (Fig. 4C).

4. Discussion

RAGE has been reported to activate some intracellular signals including the MAPK cascade and cdc42/Rac pathway leading to amplification or progression of various diseases

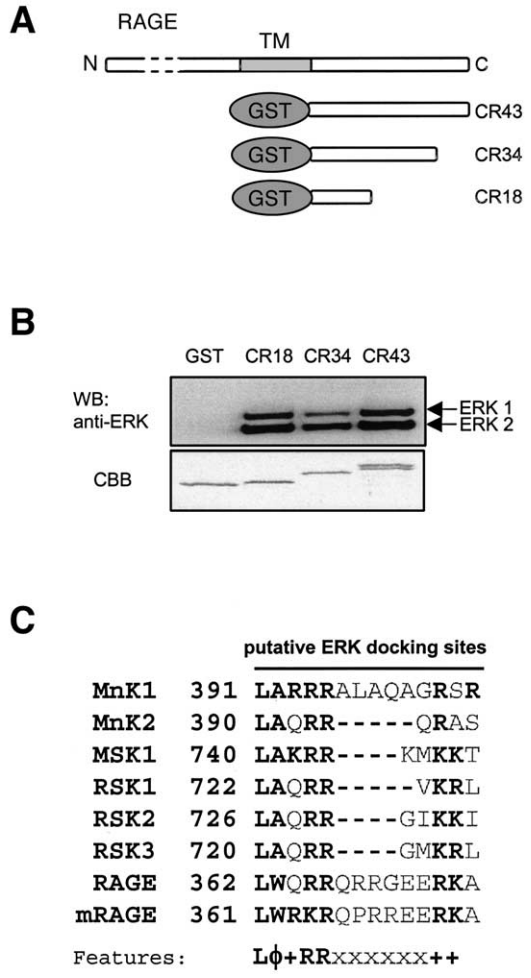

Fig. 4. The membrane-proximal region in the C-terminal cytoplasmic domain of RAGE is required for interaction with ERK-1 and -2. A: The constructs of various versions of RAGE are shown schematically. Various deletion mutants of human RAGE fused with GST were produced and affinity-purified with glutathione-Sepharose beads. N, N-terminus; C, C-terminus; TM, transmembrane. B: Rat lung extracts were mixed with glutathione-Sepharose beads with immobilized CR18, CR34, or CR43-GST (CBB, gel stained with Coomassie brilliant blue R-250). Bound proteins were eluted with buffer E, and the eluate was resolved by SDS–PAGE, followed by probing with anti-ERK polyclonal antibody, K-23 (WB, western blot). C: Sequence motif required for interaction of RAGE with ERKs. Alignment of the ERK docking sites of MnK1/2, MSK1, and RSK1, 2, and 3 with the cytoplasmic membrane-proximal regions of human and mouse RAGEs. Conserved residues within the putative binding sites are highlighted. For consensus sequence: x, any amino acid; +, basic amino acids; and ϕ, hydrophobic amino acids. NCBI accession numbers: mouse MnK1, 1929059 (EMBL, Y11091); mouse MnK2, 1929061 (EMBL, Y11092); human MSK1, 3411157 (accession, AF074393); rat RSK1, 2117822 (PIR, A53300); mouse RSK2, 125691 (SwissProt, P18654); human RSK3, 2117823 (PIR, A57459); human RAGE, 26787960 (accession, NM_001136); mouse RAGE, 6671524 (accession, NM_007425).

including diabetic vascular complications, inflammation, and tumor growth/metastasis. Concerning RAGE intracellular signaling pathways, the cytoplasmic region of RAGE is considered to be responsible for the binding of signaling molecule(s) [9,20]. However, no signaling molecule which binds directly to the RAGE cytoplasmic region has been identified yet.

In the present study, using an affinity chromatography technique, we identified ERK-1/2 as RAGE binding proteins from the rat lung extract. Direct binding capacity of the conserved ERK-2 with RAGE regardless of the phosphorylation status of ERK-2 was demonstrated between two species by the binding assay in vitro using recombinant ERK-2. Whether ERK-1 binds RAGE directly or through ERK-2 was not clarified, because the same experiment was not done with ERK-1. However, ERK-1 binding could also be important for RAGE signal transduction, because many studies have indicated that both ERK-1/2 could be involved downstream of RAGE signal transduction [21,23,31].

To confirm the interaction between RAGE and ERK in cells, immunoprecipitation and immunoblotting analysis were carried out using anti-RAGE and anti-ERK antibodies. ERK-1/2 were co-immunoprecipitated with RAGE by anti-RAGE IgG from HT1080-hRAGE cell extract. Interestingly, RAGE–ERK interactions in HT1080-hRAGE cells were detected when the cells were stimulated with amphoterin, indicating that the RAGE–ERK association depends upon the stimulation of RAGE by the ligand. Whether the binding of amphoterin to RAGE causes a conformational change of RAGE and/or its clustering, which leads to RAGE binding of ERK, is not clear in detail at present. However, it is known that the receptor clustering induced by ligand engagement is critical for the receptor activation leading to transmission of signals into the nuclei of cells [32]. Examples of this include homodimer formation of epidermal growth factor receptor [33] and oligomerization of tumor necrosis factor receptor [32–35]. In our affinity purification system using the RAGE–GST fusion protein, RAGE immobilization on the beads possibly formed a high density of RAGE providing the ERK binding site(s) which could be created by the ligand-induced activation of native RAGE in the cell.

It was also examined whether or not enhancement of ERK by a ligand treatment could be detected by measuring the kinase activity of ERK co-precipitated with the RAGE immunoprecipitates. We observed increased ERK kinase activities in RAGE immunoprecipitates from 20 to 80 min after the amphoterin treatment of cells. However, RAGE binding ERKs were detected within 5 min after the amphoterin treatment and the levels reached a maximum at 20 min, and then decreased to the same level of that at 5 min by 80 min. There was an apparent difference in the time course between the level of ERK binding with RAGE and the ERK kinase activity. It may be considered that the whole ERK binding with RAGE reached a peak early, while phospho-ERK binding or phosphorylation of ERK was still increasing up to 80 min. Although we cannot completely exclude the possibility of the presence of other kinases in addition to ERK in the immunoprecipitates, our present results strongly suggest the ligand-induced interaction of ERK with RAGE leads to the enhancement of ERK kinase activity in the cell.

We next addressed ERK binding sites in the cytoplasmic region of RAGE by in vitro binding experiments using the two deletion mutants of CR43. The primary structure of the cytoplasmic region of RAGE, which is critical for signal transduction, is conserved in various species including human, bovine, mouse, and rat. This region is further divided into at least three parts by aligning the amino acid sequences of RAGEs: a membrane-proximal 17 amino acid domain relatively rich in basic amino acids, a central 17 amino acid domain rich in glutamic acid and low-conserved C-terminus of nine residues. We found that the membrane-proximal domain of the

C-terminal cytoplasmic region of RAGE is required for the direct ERK–RAGE interaction. Recently, some ERK substrates have been shown to contain motifs that mediate high affinity interactions with ERK. Such examples are a docking site D-domain in phosphatases [36,37], transcription factors [38], phosphodiesterase [39] and kinases [40] and an FXFP motif in ETS transcription factors of the Elk subfamily [38,40]. The region required for the direct ERK–RAGE interaction has a similarity to the D-domain-like ERK docking sites of MnK1/2, MSK1, and RSKs, which are characterized by a cluster of basic residues [39–43]. Smith et al. [42] reported that carboxy-terminal tails of the RSKs contain an ERK docking site and interact with ERK but not with JNK or p38 MAPK. The number and placement of lysine and arginine residues within the conserved region correlate with the specificity for activation by ERK in vivo. We also confirmed that RAGE specifically binds with ERK but not with JNK or p38 MAPK (data not shown).

Our results and others' previous observations on ERK-interacting molecules suggest that RAGE is one of the ERK substrates. However, in the cytoplasmic region of RAGE, there is neither a conserved Ser or Thr residue nor a phosphorylation site motif, Pro-X-(Ser/Thr)-Pro which ERK can recognize. Further, ERK bound directly with RAGE regardless of its phosphorylation status. These observations suggest that RAGE is not an ERK substrate but stabilizes ERK under the proximal region of the plasma membrane and leads to activation of the interaction between ERK and its substrates. On the other hand, the cytoplasmic central region of RAGE, which was not required for binding to ERK, is highly conserved with a cluster of glutamic acid among species, suggesting the possibility of the existence of an additional binding molecule(s). However, we detected only ERK as a RAGE binding protein in our experimental conditions in vitro.

Thus, our data together with previous observations suggest that RAGE-mediated modulation of ERK [44–46], for instance (prolonged) activation of ERK, altering its subcellular localization/substrate specificity, and cross-talking with other intracellular signals, may result in sustained activation of cells and tissue dysfunction via its downstream effectors such as NF-κB [25]. Further studies will be required to examine our hypothesis that (i) ligand-induced translocation of ERKs to the plasma membrane through RAGE might enable them to be efficiently phosphorylated by MEK. Then, (ii) ERK phosphorylates other protein(s) bound on RAGE which serves as a scaffolding protein like kinase suppressor of Ras [47,48] and leads to the activation of diverse signaling.

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