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Sequencing of Two Alternatively Spliced mRNAs Corresponding to the Extracellular Domain of the Rat Receptor for Advanced Glycosylation End Products (RAGE)

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The receptor for advanced glycosylation end products (RAGE) is an integral membrane protein responsible for the recognition and internalization of those extensively modified proteins. The receptor has an extracellular domain that binds to the advanced glycosylation end products. By reverse-transcription and polymerase chain reaction amplification, we have identified in rat liver and kidney two amplified products that correspond to cDNA coding for a part of the extracellular domain of the receptor. Sequencing of these products showed that these amplified molecules were similar except for a 27-bp fragment that was absent in the smaller product. This spliced region is located close to the transmembrane region of the receptor. We have confirmed the possibility of the alternative splicing in the generation of these mRNA isoforms by cloning a fragment of the rat gene for RAGE. This fragment has a distribution of introns and exons fully compatible with the proposed alternative splicing.

Protein, lipids and nucleic acids may be covalently modified by glucose. Non enzymatic protein glycosylation due to high blood glucose levels is one of the mechanisms responsible for the long term diabetic complications (1, 2). In a first step, a Schiff base is formed between glucose and proteins. If glycemia remains high for some period, the glycosylation evolves to produce covalently and irreversible modified products termed Advanced Glycosylation End products (AGEs) (3).

These circulating AGEs are recognized by a special kind of membrane receptor located in the liver, lung, kidney, endothelium, monocytes, smooth muscle and other cells (4). Receptors for AGEs (RAGE) are able to bind and remove circulating AGEs, eliciting intracellular responses, i.e. an increase in the oxidative levels of the cells and cytokines and growth factors liberation by monocytes. These intracellular responses and the accumulation of AGEs (1) produce some of the long term complications of diabetes, such as atherosclerosis (5, 6).

The RAGE protein has been characterized as an integral protein that forms a membrane complex by interacting with a lactoferrin-like polypeptide (7, 8), although some other proteins have also been proposed as components of the complex (9). The RAGE protein belongs to the immunoglobulin superfamily of cell-surface molecules (10, 11). It has a modular structure with an extracellular domain responsible for the recognition and binding of the AGEs, a transmembrane helix and an intracellular domain responsible for the generation of the intracellular responses (12). A scheme of the RAGE domains distribution is shown in Fig. 1.

In this work, we described the identification of an alternative splicing that generated two mRNA molecules from the RAGE gene in rat liver and kidney. The spliced region is located in the extracellular domain close to the transmembrane region. We have confirmed the possibility of the alternative splicing in the generation of these mRNA isoforms by cloning a fragment of the rat gene for RAGE. In this fragment, the distribution of introns and exons is compatible with the proposed alternative splicing.

MATERIALS AND METHODS

cDNA and genomic libraries. Two cDNA libraries provided by Stratagene, La Joya, CA: an Unizap XR rat liver (Cat. No. 937507) and an Unizap XR rat kidney (Cat. No. 937503) libraries, were used for the amplification of the RAGE cDNAs. The amplification of a fragment of the RAGE gene was carried out from a Charon4A rat genomic library (13).

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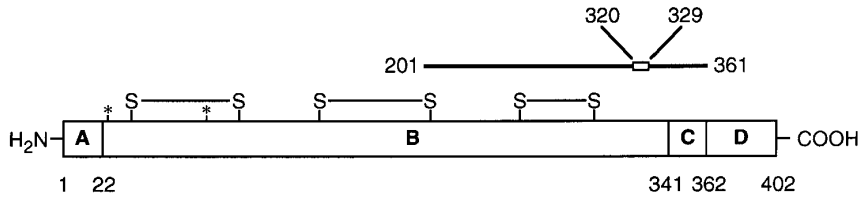

FIG. 1. Domain structure of the RAGE. The protein domains distribution shown is based on that described by (12). Numeration correspond to the deduced amino acid sequence. (A) Signal sequence. (B) Extracellular domain. (C) Transmembrane region. (D) Intracellular domain. Asterisks indicate the proposed glycosylation sites. The bar above the scheme indicates the cDNA region studied in this article. The open box within the bar represents the amino acids corresponding to the alternative splicing proposed.

Oligonucleotide primers used for amplification. The sequences of oligonucleotide primers were designed based on the rat RAGE sequence (12) as previously described for the amplification of the human sequence (14). For the amplification of a 480-bp fragment of the RAGE cDNA the oligonucleotide primers RAGE-Forward (5′-CTACCTATTCCTGCAGCTTC-3′) and RAGE-Reverse (5′-CGCCACAGGATGGCCCC-3′) have been synthesized. For the amplification of the entire coding region of the rat RAGE the oligonucleotide primers RAGE-NH_2 (5′-ATGCCAACGGGGACAGTAGC-3′) and RAGE-COOH (5′-AAGGTCCCCCTGCA CCATTC-3′) have been designed.

Amplification of reverse transcribed RNA, cDNA and genomic DNA by PCR. Total RNA from liver and kidney has been isolated by a guanidinium isothiocyanate water saturated phenol extraction method (15). First strand cDNA synthesis was performed on 5 μg total RNA in a final volume of 15 μl using a *Not*I-oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase as described by the manufacturer (Pharmacia First Strand Synthesis kit). PCR amplifications were accomplished by using a set of oligonucleotide primers as indicated above. Reaction containing no reverse transcripted samples were run to demonstrate absence of genomic DNA contamination. Reactions were performed in a DNA thermal cycler (Ericomp EasyCycler) in a 50 μl final volume of reaction buffer (50 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris–HCl, pH 9.0) containing 2.5 μl of reverse transcripted RNA or 2 μl of the rat liver or kidney cDNA libraries (1×10^8 pfu/ml), 100 pmol of each primer and 0.2 μM concentration of dNTPs. Cycling conditions were: An initial denaturation at 94°C for 5 min, denaturation at 91°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. PCR was repeated 35 cycles.

DNA sequencing and manipulations. PCR amplified products were isolated after agarose electrophoresis by electroelution (16). Isolated products were cloned by using the pGEM-T-easy cloning system (Promega Co.). Isolated fragments as well as cloned products (two independent clones per ligation) were sequenced by using an ABI 373 sequenciator. Sequencing reactions were carried out using AmpliTaq FS polymerase and fluorescent-labeled chain terminators (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit) (Perkin–Elmer). For Southern blot analysis, amplified samples were electrophoresed on agarose and alkaline transferred onto BrightStar-Plus positively charged nylon membranes (Ambion Inc., Austin, TX) according to the manufacturer instructions. Blots were hybridized with [^{32}P]-dCTP random primer labeled cDNA probes (1×10^6 cmp/mL) according to standard techniques (16).

For the amplification, identification and isolation of a fragment of the rat gene for RAGE we have used a rat genomic library. A fragment of the gene for the rat RAGE has been amplified by PCR as described above using the oligonucleotide primers RAGE-Forward and RAGE-Reverse. The amplified products have been separated on a 1% agarose gel. The band corresponding to the rat RAGE gene has been identified by southern-blot using as probe a random labelled fragment corresponding to the cDNA PCR amplified RAGE fragment. The band which gave the hybridization signal was electroeluted and cloned on the pGEM-T-easy vector system as described above. The isolated band as well as the cloned fragments were sequenced as described.

RESULTS

RNA was isolated from liver and kidney of streptozotocin-induced diabetic rats. A set of oligonucleotide primers was designed in order to quantitate the amount of mRNA coding for the rat RAGE gene by PCR (Fig. 2). These primers, RAGE-Forward and RAGE-Reverse amplified the portion shown in Fig. 1 and were designed based on the previously described oligonucleotide primers used in the amplification of a fragment of the human mRNA (14). When these primers were used for the amplification of reversed transcripted RNA from rat liver and kidney, two bands of 480 and 450 bp approximately were obtained after the separation of the amplified products on a 4% Nusieve agarose gel. A similar result was obtained when the amplification was carried out using as starting material a Unizap rat liver or kidney cDNA libraries (data not shown).

These two amplified bands were isolated by electroelution and independently cloned using a pGEM-T-easy cloning system. The sequencing of the plasmids carrying the top band yielded a 480-bp sequence that matched that described for the previously isolated rat cDNA (12) (Fig. 2). The sequencing of the plasmids containing an insert corresponding to the bottom band produced a 453-bp sequence that also matched the described sequence except for the deletion of a 27-bp fragment. The same results were obtained when the amplified, isolated fragments were sequenced directly without cloning. Therefore, these results clearly led to the possibility of the existence of isoforms corresponding to the extracellular domain of the rat RAGE.

To confirm this result, a PCR amplification of the entire coding sequence of the gene was carried out. For this purpose, a set of oligonucleotide primers termed RAGE-NH_2 and RAGE-COOH, corresponding to the amino and carboxy ends of the RAGE protein were used. From reverse transcripted liver or kidney RNA it was shown that this PCR amplification produced two close bands of a size according to the expected from the

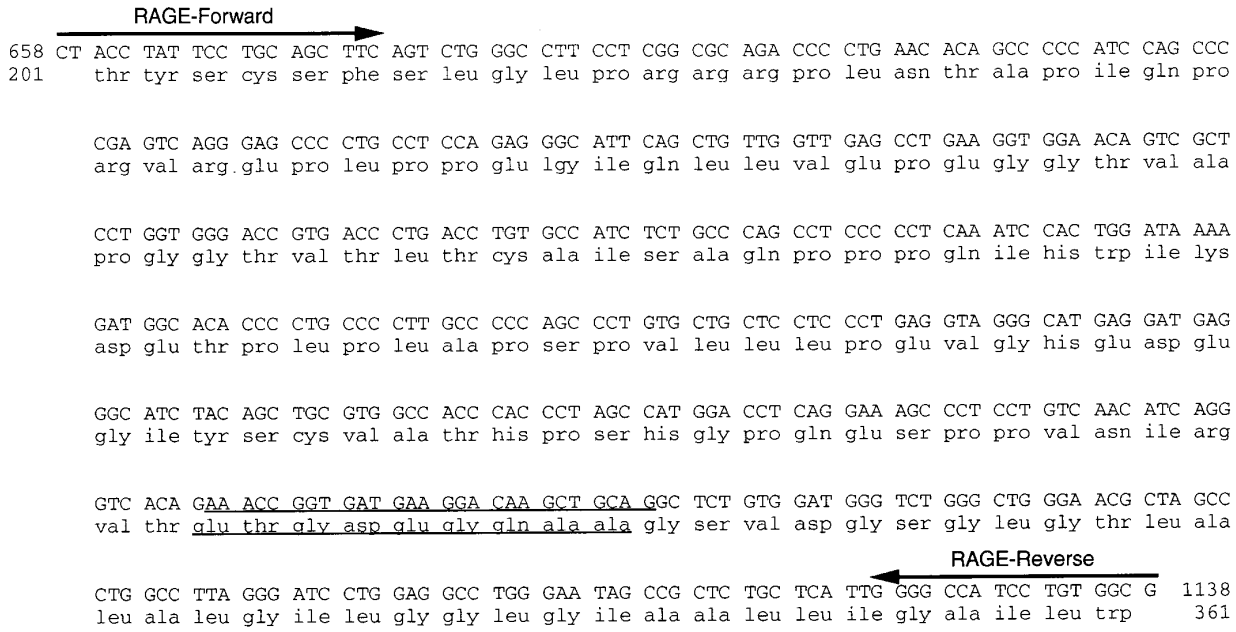

FIG. 2. Sequence of the bands amplified by PCR from the reverse transcripted RAGE mRNA. A PCR was carried out as described in material and methods using the oligonucleotide primers RAGE-Forward and RAGE-Reverse. Two bands were isolated, cloned and sequenced. Both bands shared the sequence shown except for the underlined sequence that was absent in the bottom band. DNA and deduced amino acids numeration was made according to (12).

entire coding sequence. The same result was obtained when liver or kidney cDNA libraries were used. As it was expected, the isolation and sequencing of the bottom band produced the same previously described sequence lacking the 27-bp fragment. Therefore, this result confirmed the existence of multiple forms of the RAGE mRNA, probably generated by an alternative splicing mechanism.

To test the possibility of a splicing mechanism for the generation of the different RAGE mRNA forms, the isolation and sequencing of a fragment of the gene for the rat RAGE was carried out. A PCR amplification of a genomic rat library was performed using the RAGE-Forward and RAGE-Reverse primers. The amplified products were separated on an 1% agarose gel and analyzed by southern-blot. A random primer labelled cDNA fragment corresponding to the RAGE top band previously amplified was used as probe. This southern-blot showed a strong hybridization signal corresponding to an approximately 1200-bp band. This band was isolated, sequenced and cloned on a pGEM-T-easy vector. The sequence corresponding to the isolated genomic fragment was 1193 bp long and showed a sequence compatible with the RAGE gene (Fig. 3).

By comparing the genomic isolated sequence with the sequence corresponding to the RAGE mRNA it was possible to identify the distribution of exons and introns in the fragment of the RAGE gene (Fig. 3). The exon–intron junctions matched the consensus sequences for splicing (17). All the introns started with a GT dinucleotide and ended with an AG dinucleotide. Exon 4 corresponds to the 27-bp sequence that we found absent in the bottom band amplified in our PCR. Therefore, the possibility on an alternative splicing for the generation of the mRNA isoforms is strongly supported by the distribution of introns and exons in the RAGE gene.

DISCUSSION

The RAGE is an integral protein that belongs to the immunoglobulin superfamily (11). This protein, together with a lactoferrin-like polypeptide, forms a membrane complex able to recognize and internalize AGEs (7, 8). During this process, several intracellular responses are elicited (6). The RAGE complex is related to other receptors like the macrophage scavenger receptor, which is composed of trimeric collagen-like molecules (18). This receptor is also able to bind and mediate the endocytosis of AGEs (19). All these receptors share a broad ligand specificity and polyanion sensitivity (20, 21). Therefore, there are several pathways for the internalization of AGEs in the cell. Furthermore, it has been suggested the possibility that RAGE complex could play a role in physiological processes not related with the glycosylation of proteins (10, 22).

In this article, we describe the identification of mRNA coding for two isoforms of RAGE. PCR amplification results always should be taken cautiously, however, we have taken all the following steps to ensure

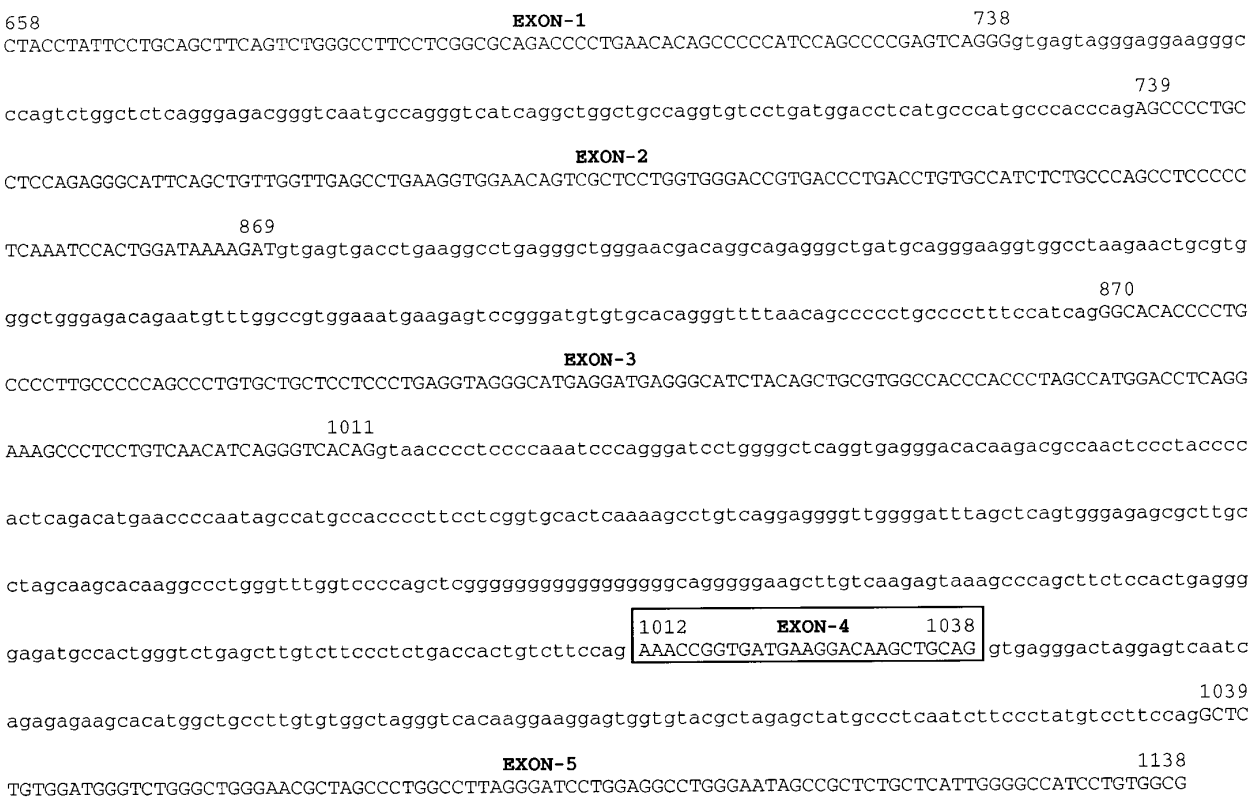

FIG. 3. Exons and introns distribution in a fragment of the rat RAGE gene. The sequence of the amplified fragment of the rat RAGE gene is shown. The exon sequences are shown in capital letters and were deduced by comparing with the sequence corresponding to the mRNA. The 27-bp exon 4 is shown in a box. Exon numbers have been assigned arbitrarily.

the validity of our results: (i) We have obtained the alternatively spliced form with reverse transcripted RNA and cDNA libraries from two different tissues, liver and kidney. (ii) The alternatively spliced form has been obtained by using two different sets of oligonucleotide primers in the PCR amplification, one of them amplifying the entire coding sequence of the RAGE. (iii) The band corresponding to the alternatively spliced form has been isolated, cloned and sequenced guaranteeing that this band obtained on the PCR amplification is not an artifact due to the formation of heterodimer duplex DNA (23). (iv) The alternatively 27-bp spliced fragment matched an exon identified from a genomic DNA fragment corresponding to the RAGE gene and therefore the possibility of an alternative splicing is strongly suggested.

The RAGE gene and protein is highly conserved in vertebrate species (11, 12). In Fig. A there is an alignment of the protein region that we have described in the rat with correspondent sequences of cow (11), mouse (12), and human (11) RAGE protein. The alternative spliced region codes for 9 amino acids. This portion is characterized by the presence of negative charged residues (Glu and Asp). In all the aligned sequences a high homology has been registered in the region studied. When the alignment is made using the described cDNA sequences (Fig. 4B) a high degree of homology is also obtained and the sequences flanking the proposed spliced fragment in the rat are highly conserved in all the species studied. Therefore, the possibility that the splicing which we have described in the rat could take place in other vertebrates, including humans, have to be taken into account.

This possibility increases when we compare the intron-exon distribution that we have found in the rat RAGE gene with that previously described for the human gene (24). On the one hand, the overall distribution of exons and introns in both species is similar. On the other hand, the rat exon 4 that we have proposed in this paper has exon 9 as its human counterpart (24). Therefore, it would be possible that an alternative splicing of the exon 9 in the human RAGE can take place.

The existence of these two forms of the RAGE, generated by an alternative splicing, could be justified by the possibility of a different specificity for glycosylated ligands and its different roles in physiological and/or pathological processes (10). Our data are not sufficient to support a theory explaining the roles of these two forms in the cell. However, two relevant facts may enhance the importance of the splicing in this region: First, the location of the spliced region in the extracel-

A

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Rat     VATHPSHGPQESPPVNIRVTETGDEGQAAGSVDGSGLGTLALALGILGG

Cow     VATHPSHGPQESRAVSVTIIETGEEGTTAGSVEGPGLETLALTLGILGG
Mouse   VATHPSHGPQESPPVSIRVTETGDEGPAEGSVGESGLGTLALALGILGG
Human   VATHSSHGPQESRAVSISIIEPGEEGPTAGSVGGSGLGTLALALGILGG
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B

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Rat     CTGTCAACATCAGGGTCACAGAAACCGGTGATGAAGGACAAGCTGCAGGCTCTGTGGATGGGTCTGGGC

Cow     CTGTCAGCGTCACGATCATCGAAACAGGCGAGGAGGGGACGACTGCAGGCTCTGTGGAAGGGCCGGGGC
Mouse   CTGTCAGCATCAGGGTCACAGAAACCGGCGATGAGGGGCCAGCTGAAGGCTCTGTGGGTGAGTCTGGGC
Human   CTGTCAGCATCAGCATCATCGAACCAGGCGAGGAGGGGCCAACTGCAGGCTCTGTGGGAGGATCAGGGC
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FIG. 4. Alignment of the protein and cDNA region alternatively spliced in rat with the cow (11), mouse (12) and human (11) RAGE sequences. (A) Protein sequences alignment. In bold the deduced amino acids sequence corresponding to the spliced region is shown. (B) cDNA sequences alignment. In bold it is shown the nucleotide sequence corresponding to the spliced region. Flanking regions are underlined.

lular domain, responsible for the recognition of ligands (12). Second, the fact that the amino acids corresponding to this region are mostly negatively charged and the glycosylation of proteins produces the removal of positive charges in the modified proteins (3). All this suggests that the isoforms of the receptor could show a modified ligand specificity and/or affinity. Apart from this, other interactions that take place in the RAGE complex, such as the binding to the lactoferrin-like peptide or the transduction of conformational changes to the intracellular domain after binding of ligands could be modified in the spliced form.

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