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FULL REPORT

The Bioinformatics and Molecular Biology Approaches for Vascular Cell Signaling by Advanced Glycation Endproducts Receptor and Small Ubiquitin-Related Modifier

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SYNOPSIS

The advanced glycation endproducts receptor (AGE-R) is a signal transduction receptor for multiligand such as S100b and AGEs. S100b has been demonstrated to activate various cells with important links to atherosclerosis initiation and progression including endothelial cells, and smooth muscle cells via AGE-R, triggering activation of multiple signaling cascades through its cytoplasmic domain. Many studies have suggested AGE-R might even participate in the cardiovascular complications involved in the pathogenesis of type I diabetes. Recently, Small Ubiquitin-Related Modifier 1 (SURM-1 also known as SUMO-1) has been recognized as a protein that plays an important role in cellular post-translational modifications in a variety of cellular processes, such as transport, transcriptional, apoptosis and stability. Computer Database search with SUMOplot Analysis program identified the five potential SURMylation sites in human AGE-R: K43, K44, K123, and K273 reside within the extracellular domain of AGE-R, and lastly K374 resides with the cytosolic domain of AGE-R. The presence of the consensus yKXE motif in the AGE-R strongly suggests that AGE-R may be regulated by SURMylation process. To test this, we decided to determine if AGE-R is SURMylated in living vascular cell system. S100b-stimulated murine aortic vascular smooth muscle cells were used for western blot analysis with relevant antibodies. Taken together, bioinformatics database search and molecular biological approaches suggested AGE-R is SURMylated in living cardiovascular cell system. Whilst SURMylation and AGE-R undoubtedly plays an important role in the cardiovascular biology, it remains unclear as to the exact nature of this contribution under both physiological and pathological conditions.

	human AGE-R		murine AGE-R
K43	KCKGAP**K**KP PQRLE	**K43**	SCKGAP**K**KP PQQLE
K44	CKGAP**K**KPP QRLEW	**K44**	CKGAP**K**KPP QQLEW
K123	VYQIP**GK**PE IVDSA	**K122**	VYQIP**GK**PE IVDPA
K173	GKETL**VK**EE TRRHP		
K273	PQIHW**MK**DG VPLPL	**K272**	PQVHW**IK**DG APLPL
K374 (tail)	RRGEE**RK**AP ENQEE	**K373 (tail)**	PRREE**RK**AP ESQED

Key Words: AGE-R; SURMylation; SURM-1; S100b; cardiovascular

INTRODUCTION

The advanced glycation endproducts receptor (AGE-R) is a member of the immunoglobulin (Ig) superfamily of cell surface molecules[1-3]. The receptor is composed of an extracellular region containing one "V"-type and two "C"-type Ig domains. This is followed by a hydrophobic transmembrane-spanning domain which in turn neighbors a highly charged, short cytoplasmic domain that is essential for post-AGE-R signaling[1-3]. This has been termed "full-length" or membrane AGE-R. In addition, a number of isoforms have been identified. AGE-R was initially identified and characterized for its ability to bind advanced glycation end products (AGEs), adducts formed by glycoxidation that accumulate in disorders such as diabetes. Subsequently, AGE-R has also been shown to be a pattern recognition receptor, recognizing families of ligands rather than a single polypeptide. Such ligands include amyloid fibrils, amphoterins, S100/calgranulins, and Mac-1[1-3].

The ligands of AGE-R such as S100B have been demonstrated to activate various cells with important links to atherosclerosis initiation and progression including endothelial cells (EC), smooth muscle cells (SMC) and peripheral blood mononuclear cells (PBMC) via AGE-R, triggering activation of multiple signaling cascades through its cytoplasmic domain, including p21ras, erk1/2 (p44/p42) MAP kinases, p38 and SAPK/JNK MAP kinases, rho GTPases, phosphoinositol-3 kinase and the JAK/STAT pathway and downstream consequences such as activation of Nuclear Factor (NF)-kB and CREB, and generation of cytokines and proinflammatory molecules[2]. In this context, AGE-R blockade halts the progression of established atherosclerosis in apoE null mice and impact in diabetes[1-3]. Blockade of AGE-R in euglycemic mice suppressed the challenge phase of delayed type hypersensitivity (DTH) in response to methylated BSA, diminished colonic inflammation in mice deficient in IL-10, and decreased phenotypic and molecular indices of arthritis in DBA/1 mice subjected to sensitization/challenge with bovine type II collagen[3]. We speculate that S100/calgranulins, together with AGEs, amplify proinflammatory mechanisms in the vessel wall, especially in diabetic cardiovascular complications[1-3].

In the majority of healthy adult tissues, AGE-R is expressed at a low basal level. Many lines of evidence have suggested that the expression of AGE-R (receptor for advanced glycation end products) is upregulated in human tissues susceptible to the long-term complications of diabetes[2]. From the kidneys to the macrovessels of the aorta, AGE-R expression is upregulated in a diverse array of cell types, from glomerular epithelial cells (podocytes) to endothelial cells, vascular smooth muscle cells, and inflammatory mononuclear phagocytes and lymphocytes. This suggests that the ligand-AGE-R interaction propagated inflammatory mechanisms linked to chronic cellular perturbation and tissue injury. Indeed, such considerations suggested that AGE-R might even participate in the pathogenesis of type 1 diabetes[2]. Our previous studies have shown that pharmacological and/or genetic deletion/mutation of the receptor attenuates the development of hyperglycemia in NOD mice, in mice with myriad complications of diabetes, interruption of ligand-AGE-R interaction prevents or delays the chronic complications of the disease in both macro- and microvessel structures[2]. These findings suggest that AGE-R is "at the right place and time" to contribute to the pathogenesis of diabetes and it complications. Studies are in progress to test the premise that antagonism of this interaction is a logical strategy for the prevention and treatment of cardiovascular complications in diabetes[1-3].

Recently, much attention has been paid to protein post-translational modifications (PTMs) in cellular process, also called as covalent modifications, which play important role in all kinds of biological processes and pathways. To date, there were more than 350 types of PTMs discovered. However, only a few of them were well-studied, eg., phosphorylation, glycosylation, ubiquitination and SURMylation, etc.[4,5]. Besides experimental methods, numerous computational predictors were developed and popular for their convenience, accuracy, and fast-speed. The computational tools are generally classified into two groups based on their PTMs types, including Attachment of chemical groups and Peptide cleavage/Protein sorting/subcellular localization[4,5]. While we roughly examined the amino acid sequence of AGE-R to study the cellular physiological role of AGE-R, we interestingly identified a couple of the consensus SURMylation motif in AGE-R amino acid sequences. The systematic computer data base search tool analysis indicated that AGE-R has the consensus SURMylation motif, defined as yKXE, where y represents a hydrophobic residue (such as leucine, isoleucine, valine or alanine), K represents the lysine residue targeted for SURMylation, X represents any amino acid and E represents glutamic acid. Although the same lysines may putatively be targets for ubiquitination, unlike ubiquitination, the SURM modification does not usually lead to degradation of the protein. Indeed, in some cases, SURMylation may provide a modification to prevent degradation, such as has been discovered in the case of IkB alpha[4,5]. The small ubiquitin-related modifier has been known to play a key role in diverse processes such as nuclear protein import, protein targeting to and formation of certain subnuclear structures, and the regulation of a variety of processes such as the inflammatory response in mammals. More specifically, 1) Cytosol/Nucleus Trafficking via Nuclear Pore Channel (NPC) (Translocation) RANGAP, RANBP2, 2) Regulation of transcription factor STAT1, c-Jun, c-Myb, Sp3, IRF-1, SREBPs, SRF, Elk, AP1, AP2, androgen receptor (AR), glucocorticoid receptor (GR), and progesterone receptor (PR), 3)

Inhibitor of ubiquitin-mediated degradation and stabilization of bound protein-CREB, 4) DNA damage repair and regulation of genomic stability, and 5) Mitochondrial fission[4,5]. The process of SURMylation results in lysine modifications of proteins by the addition of a polypeptide group to the backbone protein. SURMylation of proteins involves at least three central steps, The Sumo activating enzyme E1 transfers activated SURM to the E2-conjugating enzyme Ubc9 that directly catalyzes the isopeptide bond formation between the C-terminal glycine residue of the activated SURM to the lys residue of the target protein. E3 SURM ligases then attach SURM to the target protein[4,5].

In this study, the powerful bioinformatics analysis allowed us to identify the conserved SURMylation site motif at AGE-R. To explore its biological role in cardiovascular system, the potential interactions of AGE-R and SURM1 were tested in vascular cell culture.

RESULTS AND DISCUSSION

As described above, SURMylation is a post-translational modification event in cells that may alter various cellular properties[4,5]. It has been well established that AGE-R plays an important role in vascular cells[1-3]. We therefore speculated that AGE-R might be SURMylated in vascular cell system. Before we determine if AGE-R is SURMylated in living cells, we first utilized the bioinformatic approaches using the six different SURMylation computational predictor database searches as described in materials and methods section. Among these database searches, we selected the computer database search data generated from The SUMOplot™ Analysis program to determine if putative SURMylation sites were present in AGE-R. As shown in Table 1-(A), the five conserved potential SURMylation sites were identified in human AGE-R: K43, K44, K123, and K273 reside within the extracellular domain of AGE-R, and lastly K374 resides with the cytosolic domain of AGE-R in SUMOplot™ within the range of score 0.67-0.34 in human AGE-R and range of score 0.93-0.34 in mouse AGE-R. Thus, sites for potential SURMylation reside within the ligand binding region (first four) and component of AGE-R essential for modulation of cellular signaling (fifth potential site in the cytosolic domain). These sites are exactly conserved in murine AGE-R as well in Table 1-(B), suggesting that SURMylation is an important mechanism of regulation across the species. Interestingly, murine AGE-R contains one additional potential SURMylation site (K173 in the extracellular domain).

The conserved yKXE motifs of K43, K44, K123, and K173 in the extracellular domain and K374 in the intracellular domain were aligned and highlighted in bold characters and underlined in Table 2, clearly showing the presence of conserved yKXE motif in AGE-R among species.

The presence of the consensus yKXE motif in the AGE-R strongly suggests that AGE-R may be regulated by SURMylation process in vascular system. We speculated that AGE-R might be SURMylated in living vascular cells. To determine if AGE-R is SURMylated in vascular cell culture, mouse aorta vascular smooth muscle cells (SMC) were incubated with its ligand S100b for 0, 20 min or 10 hr. The ligand S100b-stimulated cell lysates were immunoprecipitated with anti-mouse AGE-R antibodies and immunoblotted with anti-SURM-1 antibodies and visualized by ECL. As indicated in Figure 1 (lane 1 and lane 2),

Table 1. The SUMOplot computer database search of human AGE-R (swisspro Q15109) protein sequence (A) and mouse AGE-R (swisspro Q62151) protein sequence (B).

(A) Human AGE-R

Protein ID: gi|2497317|sp|Q15109.1|RAGE_HUMAN
Length: 404 aa

```
  1 MAAGTAVGAW VLVLSLWGAV VGAQNITARI GEPLVLKCKG APKKPPQRLE
 51 WKLNTGRTEA WKVLSPQGGG PWDSVARVLP NGSLFLPAVG IQDEGIFRCQ
101 AMNRNGKETK SNYRVRVYQI PGKPEIVDSA SELTAGVPNK VGTCVSEGSY
151 PAGTLSWHLD GKPLVPNEKG VSVKEQTRRH PETGLFTLQS ELMVTPARGG
201 DPRPTFSCSF SPGLPRHRAL RTAPIQPRVW EPVPLEEVQL VVEPEGGAVA
251 PGGTVTLTCE VPAQPSPQIH WMKDGVPLPL PPSPVLILPE IGPQDQGTYS
301 CVATHSSHGP QESRAVSISI IEPGEEGPTA GSVGGSGLGT LALALGILGG
351 LGTAALLIGV ILWQRRQRRG EERKAPENQE EEEERAELNQ SEEPEAGESS
401 TGGP
```

No.	Pos.	Group	Score
1	K123	VYQIP **GKPE** IVDSA	0.67
2	K273	PQIHW **MKDG** VPLPL	0.63
3	K43	KCKGA **PKKP** PQRLE	0.50
4	K44	CKGAP **KKPP** QRLEW	0.37
5	K374	RRGEE **RKAP** ENQEE	0.34

(B) Mouse AGE-R

Protein ID: gi|2497318|sp|Q62151.1|RAGE_MOUSE
Length: 403 aa

```
  1 MPAGTAARAW VLVLALWGAV AGGQNITARI GEPLVLSCKG APKKPPQQLE
 51 WKLNTGRTEA WKVLSPQGGP WDSVAQILPN GSLLLPATGI VDEGTFRCRA
101 TNRRGKEVKS NYRVRVYQIP GKPEIVDPAS ELTASVPNKV GTCVSEGSYP
151 AGTLSWHLDG KLLIPDGKET LVKEETRRHP ETGLFTLRSE LTVIPTQGGT
201 THPTFSCSFS LGLPRRRPLN TAPIQLRVRE PGPPEGIQLL VEPEGGIVAP
251 GGTVTLTCAI SAQPPPQVHW IKDGAPLPLA PSPVLLLPEV GHADEGTYSC
301 VATHPSHGPQ ESPPVSIRVT ETGDEGPAEG SVGESGLGTL ALALGILGGL
351 GVVALLVGAI LWRKRQPRRE ERKAPESQED EEERAELNQS EEAEMPENGA
401 GGP
```

No.	Pos.	Group	Score
1	K173	GKETL **VKEE** TRRHP	0.93
2	K272	PQVHW **IKDG** APLPL	0.77
3	K122	VYQIP **GKPE** IVDPA	0.67
4	K43	SCKGA **PKKP** PQQLE	0.50
5	K44	CKGAP **KKPP** QQLEW	0.37
6	K373	PRREE **RKAP** ESQED	0.34

Table 2. Amino acid sequence of the human and murine AGE-R (RAGE) were analyzed in SURMylation Sequence site database. The potential SURMylation sites among human and murine AGE-R were aligned, highlighted and underlined below

	human AGE-R		murine AGE-R
K43	KCKGA**PKKP** PQRLE	K43	SCKGA**PKKP** PQQLE
K44	CKGAP**KKPP** QRLEW	K44	CKGAP**KKPP** QQLEW
K123	VYQIP**GKPE** IVDSA	K122	VYQIP**GKPE** IVDPA
K173	GKETL**VKEE** TRRHP		
K273	PQIHW**MKDG** VPLPL	K272	PQVHW**IKDG** APLPL
K374	RRGEE**RKAP** ENQEE	K373	PRREE**RKAP** ESQED
(tail)		(tail)	

the 65 kDa band corresponds to the size of mouse AGE-R proteins covalently bound to SURM-1. The 55 kDa band appears to be similar size to mouse heavy chain IgG (Figure 1 lane 1-3). Whereas very little 65 kDa AGE-R appears SURMylated in the absence of S100b (20 min) (Figure 1 lane 2), increasing exposure (10 hr) to S100b results in enhanced SURMylation of AGE-R (Figure 1 lane 3). Pull-down immunoprecipitation with nonimmune IgG was negative (not shown).

To further investigate that this is cell-type specific phenomenon, the native Chinese Hamster Ovary (CHO) cells (Figure 2, lanes 1-4) and CHO cells stably transfected to express full-length human AGE-R (Figure 2, lanes 5-8) were exposed to ligand S100b for 0 hr (lanes 1&5), 5 hr (lanes 2&6), 7 hr (lanes 3&7), or 10 hr (lanes 4&8). To minimize the visualization of heavy chain IgG, anti-human AGE-R antibodies were cross-linked to protein-A sepharose beads. Following incubation, immunoprecipitation with anti-human AGE-R IgG which is cross-linked to protein A-sepharose beads was performed followed by immunoblotting with anti-SURM-l IgG. The 65 kDa protein bands corresponding to the size of AGE-R covalently bound to SURM-1 was detected. A time-dependent increase in AGE-R SURMylation was evident (Figure 2 lane 1-4) that was enhanced in full-length AGE-R expressing CHO cells (Figure 2 lanes 6-8). Pull-down immunoprecipitation with nonimmune IgG was negative (not shown). These results suggest that AGE-R is SURMylated in living cell system including mouse vascular SMC as well as CHO cells.

In this context, AGE-R has multiple potential SURMylation sites K43, K44, K123, K273, and K374. Using these site mutants, we will be able to explore the impact of interaction of AGE-R and SURM-1 in SMC biology and its potential role in the pathogenesis of cardiovascular complications in diabetes in the near future.

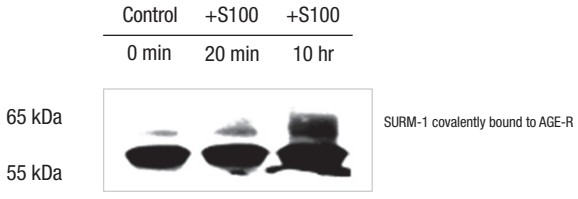

Figure 1. S100-mediated SURMylation of AGE-R in murine SMC. S100-stimulated cell lysates immunoprecipitated with anti-mouse AGE-R antibodies and immunoblotted with anti-SURM1 antibodies and visualized by ECL.

CONCLUSION AND PROSPECTS

In this study we employed a computer database search and molecular biological approach to dissect vascular cell signaling by SURM-1 and AGE-R. This study provided a good example of a power of complementary bioinformatics and molecular biology approaches to accelerate to understand the mechanism of vascular disease and biology. Furthermore, this study also clearly suggests that AGE-R is SURMylated in living vascular cell system, which appears to be closely implicated in cardiovascular complications in diabetes. Future studies will be needed to further understand its physiological role in vascular biology and disease complications.

MATERIALS AND METHODS

Computer database search for SURMylation

We have utilized the six different SURMylation computational predictor database searches for this study: (1) SUMOplot™: The SUMOplot™ Analysis Software Program predicts and scores SURMylation sites in target protein The SUMOplot™ Analysis Program predicts and scores SURMylation sites in target protein. The presence of this post-translational modification help explain larger molecular weights than expected on SDS gels due to attachment of SURM protein (11 kDa) at multiple positions in target protein. SURM-1 (small ubiquitin-related modifier, also known as SUMO-1, PIC1, UBL1, Sentrin, GMP1, and Smt3) is a member of the ubiquitin and ubiquitin-like superfamily. Most SURM-modified proteins contain the tetrapeptide motif B-K-x-D/E where B is a hydrophobic residue, K is the lysine conjugated to SUMO, x is any amino acid (aa), D or E is an acidic residue. The SUMOplot™ Analysis Program predicts the probability for the SURM consensus sequence (SURM-CS) to be engaged in SURM attachment (http://www.abgent.com/

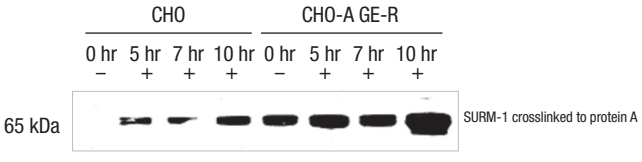

Figure 2. S100-mediated SURMylation of AGE-R in the native CHO cells and CHO cells expressing AGE-R. S100-stimulated cell lysates were immunoprecipitated with anti-human AGE-R antibodies previously cross-linked to protein A-sepharose beads and immunoblotted with anti-SURM1 antibodies and visualized by ECL.

tools/). The SUMOplot™ score system is based on two criteria: (1) direct amino acid match to SURM-CS. (2) substitution of the consensus amino acid residues with amino acid residues exhibiting similar hydrophobicity[3,6], SUMOsp 1.0: SURMylation Sites Prediction, based on a manually curated dataset, integrating the results of two methods, GPS and MotifX, which were originally designed for phosphorylation site prediction[7], (3) SUMOsp 2.0: SUMOsp 2.0 web server will be more efficient for SURMylation sites prediction[8], (4) SUMOpre: A novel method for high accuracy SURMylation site prediction from protein sequences[9], (5) SSP 1.0: SURMylation Sites Prediction, based on a manually curated dataset, integrating the results of two methods, GPS and MotifX, which were originally designed for phosphorylation site prediction[10], (6) PSFS-SUMO: Predicting the protein SUMO modification sites based on Properties Sequential Forward Selection (PSFS)[11].

Cells

The native CHO (Chinese Hamster Ovary, CCL-61™) cells were purchased from ATCC (Manassas, VA). The CHO-K1 cell line was derived as a subclone from the parental CHO cell line initiated from a biopsy of a *Cricetulus griseus* Chinese hamster female ovary and have adherent epithelial-like morphology. The base medium for this cell line was F-12K Medium. To make the complete growth medium, the final concentration of 10% fetal bovine serum at 37.0°C was added. A subcultivation ratio of 1:5 was used. Medium was renewed once or twice between subculture. Prescreening western blot test suggest that CHO cells did not show any detectable endogenous level of AGE-R background (data not shown). The CHO-K1 Chinese Hamster Ovary Cells were stably transfected with full length human AGE-R wild type cDNA (A Schmidt, New York, NY) using Lipofectamine™ (Invitrogen) and selected for a stable clone (HA-CHO). The SMC (smooth muscle cells) were isolated from murine (C57BL/6J A Schmidt, New York, NY) aortic blood vessel tissue[2,12]. The purity of isolated SMC was confirmed by staining with smooth muscle actin. Isolated SMC did not show PECAM-1 staining, excludes the possibility of contamination of Endothelial cells. The CHO and SMC cells were maintained in in Dulbecco's modified Eagle's medium (Invitrogen GIBCO BRL, CA) supplemented with 10% fetal bovine serum (Invitrogen GIBCO BRL, CA) in the presence of 5% CO_2. Cells were synchronized when cell density became 80-90% and then incubated with S100b.

Chemicals & Antibodies

S100b were purchased from Calbiochem Merck Millipore (San Diego, CA). Mouse Anti-SURM1 (SUMO1) antibodies and Rabbit anti-Ubc9 antibodies were purchased from Invitrogen Zymed (San Francisco, CA). Rabbit and Mouse Anti-AGE-R (RAGE) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Triton X-100, Tween 20, Tris, NaCl and SDS were purchased from Sigma (St Louis, MO). Fetal bovine serum, Dulbecco's modified Eagle's medium, 1X PBS (phosphate-buffered saline) were purchased from Invitrogen GIBCO BRL (Carlsbad, CA), nitrocellulose membranes were purchased from GE Healthcare Life Sciences Amersham), Anti-goat peroxidase-conjugated secondary antibody in block solution were purchased from Santa Cruz Biotechnology. Mouse monoclonal Anti-human AGE-R (RAGE) full length antibodies were purchased from Abcam (Cambridge, MA), Protein-A Sepharose beads, Goat Polyclonal Anti-human AGE-R (RAGE) (c-terminal) antibodies, horseradish peroxidase (HRP) conjugate IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Loading dye and molecular weight markers were purchased from Invitrogen (Carlsbad, CA), 10% SDS-PAGE and Immunoblot system were purchased from Invitrogen (Carlsbad, CA), enhanced chemiluminescence (ECL) system was purchased from Pierce (Rockford, IL), Hyperfilm ECL film was purchased from GE Healthcare Life Sciences Amersham (Piscataway, NJ).

Western blotting

CHO cells expressing full length AGE-R were incubated with S100b for various times (0 hr, 5 hr, 7 hr, 10 hr) at 37°C. Cells were lysed with cold 1% Triton-X100 lysis buffer, and lysate supernatant was immunoprecipitated with goat anti-AGE-R antibodies coupled to proteinA-Sepharose at 4°C. Immunoprecipitation protein A-sepharose complex was washed with lysis buffer for three times and boiled for 5 min in SDS-sample buffer and protein extracts were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare Life Sciences Amersham). Membranes were blocked with 3% (w/v) fat-free milk in 1X TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% tween-20), then incubated with the mouse antiserum directed against SURM-1 in a blocking solution for 2 hr at room temperature. Membranes were then incubated with an anti-goat peroxidase-conjugated secondary antibody in block solution (Santa Cruz Biotechnology). Each incubation steps were followed by 3 washes for 15 min in 1XTBS containing 0.1% Tween 20. The membrane was treated with ECL kit (GE Healthcare Life Sciences Amersham) and visualized in the Hyperfilm ECL (GE Healthcare Life Sciences Amersham).

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