Signaling Interface of Advanced Glycation Endproducts Receptor and Ubiquitin-Conjugating Enzyme Ubc9 Complex in Atherosclerosis and Cancer Cells

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SYNOPSIS

The advanced glycation endproducts receptor (AGER) is a multiligand signal transduction receptor. One of its ligands, S100b molecules activates vascular smooth muscle cells and endothelial cells via its receptor, thus triggering activation of signaling cascades and generation of cytokines and proinflammatory molecules. Ubiquitin-conjugating enzyme Ubc9 is an E2 conjugating enzyme that transfers the activated small ubiquitin-related modifier to protein substrates, and thus it plays a critical role in SURMylation-mediated cellular pathways. Previous studies have shown that both AGE-R and Ubc9 play roles in diverse cellular signaling pathways. However, until recently, little attention has been paid to interactions between AGE-R and Ubc9. In this study, sequence database searches allowed us to identify a potential interaction motif between AGE-R and Ubc9. The subsequent biochemical and molecular biological analysis suggested that there may be specificity in AGE-R and Ubc9 complex signaling in atherosclerosis and cancer cells in a cell-type specific manner. Although the determinant for specificity in AGE-R and Ubc9 complex signaling in cancer cells and atherosclerosis is yet to be determined, this study provides the basis to develop a specific therapeutic application of AGE-R, SURM (small ubiquitin-related modifier)-1, and Ubc9 complex activation pathways in atherosclerosis, diabetes, cancer and inflammatory diseases.
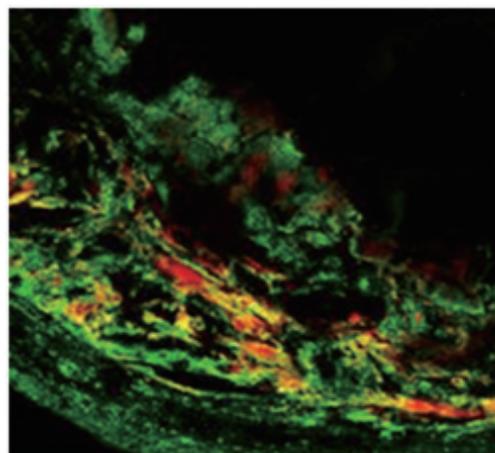
Key Words: AGE-R; SURM-1; vascular signaling; cervix; glioma; breast cancer

INTRODUCTION

AGE-R is a signal transduction receptor for S100b, which have important intracellular properties, where their roles are linked to homeostatic properties, such as calcium binding. S100b protein is imbued with EF-hand calcium-binding motifs. S100b is localized in the cytoplasm and/or nucleus of a wide range of cells, and is involved in the regulation of a number of cellular processes1. Once released into the extracellular milieu, S100b molecules activate smooth muscle cells (SMC), and endothelial cells (EC) via AGE-R, thus triggering activation of signaling cascades and generation of cytokines and proinflammatory molecules[1]. S100b may function by stimulating Ca^{2+} fluxes, neurite extension, proliferation of melanoma cells, inhibition of PKC-mediated phosphorylation and inhibition of microtubule assembly[1]. Chromosomal rearrangements and abnormal expression of this gene have been implicated in several neurological, neoplastic, and other types of diseases, including Alzheimer's disease, melanoma, epilepsy, amyotrophic lateral sclerosis, Down's syndrome and type I diabetes[1] (http://www.ncbi.nlm.nih.gov/gene/6285). Blockade of AGE-R in euglycemic mice suppressed the challenge phase of delayed-type hypersensitivity 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[1]. We speculated that S100, enriched in atherosclerotic plaques and expanding neointima after acute arterial injury, together with AGE-Rs, amplify proinflammatory mechanisms in the vessel wall, especially in diabetes.

Ubc9 is an E2 conjugating enzyme that transfers the activated SURM to protein substrates, and thus it plays a critical role in SURMylation-mediated cellular pathways[2-5]. The interaction between SURM-1 and Ubc9 is much stronger than that between ubiquitin and Ubc9 or SURM-1 and three other E2s. Furthermore, the conserved C-terminal Gly-Gly residues of SURM-1 are required for the high affinity interaction, suggesting that SURM-1 could form a thiol ester bond with Ubc9[6-8]. This is further supported by an *in vitro* assay in which a beta-mercaptoethanol-sensitive SURM-1 - Ubc9 conjugate was observed[7]. Therefore, Ubc9 is likely to be a key conjugating enzyme for the SUR-Mylation pathway[9]. Ubc9 is a multifunctional protein that enhances cell invasion and metastasis[10]. It enhances tumor growth in the xenograft mouse model using MCF-7 cell line in part through regulation of Bcl-2 expression[10]. Overexpression of wild type Ubc9 promotes cell invasion and metastasis[10]. These observations suggest that Ubc9 may be used in cancer intervention therapy because it is upregulated in various types of tumors[11-14].

In this study, we examined the potential interactions between AGE-R and Ubc9 in cancer cell lines and investigated the potential relationship between expression and distribution of Small Ubiquitin-Related Modifier-1 (SURM-1; Ubc9 binding protein; UBP), Ubc9 and AGE-R in the case of a hypothetical patient suffering from atherosclerosis.

RESULTS AND DISCUSSION

Identification of the conserved potential Ubc-9 binding motif at the C-terminal of AGE-R

Amongst the S100 calcium-binding protein, S100b is one of the AGE-R's multi-ligands[1]. S100b activates smooth muscle cells (SMC), endothelial cells (EC) and peripheral blood mononuclear cells (PBMC) via AGE-R, thus triggering activation of signaling cascades, and generation of cytokines and proinflammatory molecules[1]. However, its underlying mechanisms are still not clear. To elucidate this molecular mechanism via AGE-R, we examined whether AGE-R has any specific amino acid sequence motif with previously known functions. Computer database sequence analysis showed that the AGE-R (319-369) domain was 77% aligned to the tumor suppressor E2A (478–528) domain (see Figure 1). This E2A (478–528) domain was previously shown to be the conserved potential binding site of SURM conjugating enzyme, Ubc9[15,16]. AGE-R (319-369) and E2A (478–528) domain is commonly rich in hydrophilic DEST (D: Aspartic acid; E: Glutamic acid; S: Serine; and T: Threonine) residues. These results suggested that the AGE-R C-terminal (319-369) domain might have a Ubc9 binding capacity (see Figure 1; the DEST residues are highlighted in yellow). In addition,
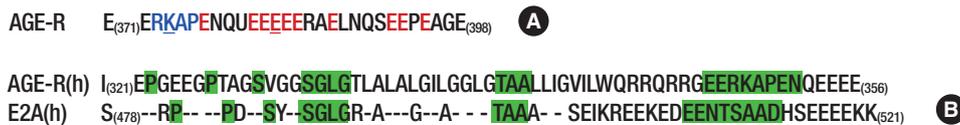

Figure 1. Sequence alignment of the conserved Ubc-9 binding motif at the AGE-R. Glutamic acid-rich region (GARR) is present at AGE-R c-terminal just near the conserved SURMylation motif. The consensus site for Ubc-9 binding and SURMylation KxE (Lys K374) are glutamic acid rich region (E383) at AGE-R. The conserved Ubc-9 binding motif was highlighted in red. The SURM-1-modified Lys (K) as well as the absolutely conserved Glu. AGE-R is aligned so as to fit the consensus sequence, which suggests that K374 is the primary modification site in AGE-R. (A) The consensus Ubc-9 binding motif and SURMylation KxE (K374) motif in human AGE-R protein. The SURMylation KxE (K374) motif was highlighted in blue block letters. The consensus Ubc-9 binding motif was highlighted in red block letters. (B) Sequence Alignment of the conserved Ubc9 binding motif in AGE-R and E2A. Ubc9-binding motif of E2A is highlighted in green and yellow. AGE-R sequence which was aligned with E2A sequence was also highlighted in green and yellow. Alignment score = 77%.

this DEST motif at AGE-R is neighbored by the consensus site for SURMylation, KxE motif (K374). Here, an aliphatic amino acid and lysine (K) is conjugated to SURM-1, which is well established as a Ubc9 binding protein (UBP)[12,13].

The Tumor Suppressor E2A is a transcription factor that plays a major role in determining the fate of tissue-specific cells during embryogenesis, such as muscle or early B-cell differentiation. Heterodimers between E2A and tissue-specific basic helix-loop-helix (bHLH) dimers bind DNA on E-box motifs: 5'-CANNTG-3'. Deletions in E2A have been observed in many cancer cell lines and a subset of pre-B-cell acute lymphoblastic leukemia (B-ALL) cases (http://www.phosphosite.org P15923). While E2A functions as a tumor suppressor, Ubc9 is known to play a role in tumor growth[10,17]. Ubc9 is a single copy gene and is ubiquitously expressed in all human organs and tissues. However, it is often upregulated in tumor specimens. Microarray analysis shows that Ubc9 mRNA is overexpressed in lung adenocarcinoma[12]. The semi-quantitative RT-PCR analysis and immunohistochemistry detected overexpression of Ubc9 in ovarian carcinoma compared to the matched normal ovarian epithelial cells[12,14]. Furthermore, it is the most highly expressed protein in extracts taken from melanoma infiltrated lymph nodes[13]. Ubc9 is able to induce Bcl-2 expression in the breast cancer cell line MCF-7[18], which could explain in part why ectopic expression of Ubc9 enhances tumor growth, while suppression of Ubc9 function reduces tumor growth in MCF-7 model[12]. Unfortunately, little is known whether or not Ubc9 can promote cell invasion and tumor metastasis. This leads us to speculate that the AGE-R C-terminal may be involved in a Ubc9 complex signaling pathway in cancer cell lines. We have decided to investigate this further by examining the manner in which Ubc9 interacts with AGE-R in cancer cells.

Cell-type specific association of Ubc9 with AGE-R immunocomplex in cancer cells

To determine whether AGE-R and Ubc9 proteins are associated in a living cancer cell culture system, HELA (human cervical cancer cells), MDA-MB-231 (human breast adenocarcinoma cells) and C6 (Rat glioma cells) were lysed and immunoprecipitated with anti-AGE-R antibody and resolved in SDS-PAGE. The membrane was blotted with anti-Ubc9 antibody and visualized by ECL. AGE-R expressing Chinese Hamster Ovarian (CHO) cells were also used as a control. In this western blot analysis, the 18 kDa protein bands were shown in human cervix adenocarcinoma epithelial cells (HeLa; Figure 2 lane 1) and rat C6 glioma cells (Figure 2 lane 3), which correspond to the size of Ubc9 protein, but this 18 kDa band was not shown in human breast adenocarcinoma epithelial cells MDA-MB-231 (Figure 2 lane 2). The 55 kDa band corresponded to the size of heavy IgG. Pull-down IPT with nonimmune IgG was not shown. In a con-
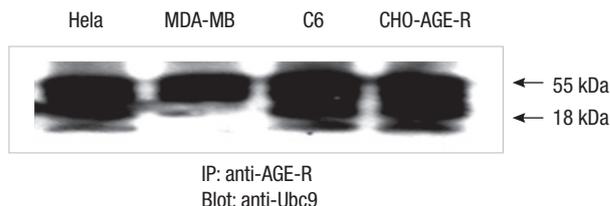

Figure 2. Specificity in association with Ubc9 proteins and the AGE-R immuno-complex in the cancer cells. We tested whether Ubc9 proteins are associated with anti-AGE-R antibody immunocomplex in cervix cancer, breast cancer and brain cancer cells by western blot analysis: Human cervix adenocarcinoma epithelial cells (Hela; lane 1), Human breast adenocarcinoma epithelial cells (MDA-MB-231; lane 2) and Rat C6 glioma cells (lane 3). As a control, this western blot analysis was tested with Chinese Hamster Ovarian (CHO) cells expressing AGE-R (lane 4). S100-treated cells (Hela, MDA-MB, C6 cells and control CHO cells expressing AGE-R) were lysed and resolved in SDS-PAGE. The membrane was blotted with anti-Ubc9 antibody and visualized by ECL. (Hela: human cervical cancer cells, MDA-MB: breast adenocarcinoma cells, C6: Rat glioma cells).

trol experiment with CHO cells expressing full length AGE-R, the 18 kDa band was shown in the western blot (Figure 2 lane 4), the same as can be seen in Figure 2 lanes 1 & 3. Thus, full length AGE-R expressing CHO cells display binding of AGE-R to Ubc9. Ubc9 was ubiquitously expressed in all of these 4 cell types above (not shown). These results suggest that Ubc9 might be associated with AGE-R in cervical cancer and brain glioma cancer cells, but not in breast cancer cells. Furthermore, these outcomes suggest that Ubc9 is in the same immunocomplex with AGE-R in cancer in a cell-type specific manner, more specifically cervical adenocarcinoma epithelial cells, but not in breast adenocarcinoma epithelial cells. Although the characterization of these two adenocarcinoma epithelial cell types is the same, the association of the Ubc9 in the same immunocomplex with AGE-R was differentially regulated depending on the original of organ source. To be sure, more cell types still need to be examined. However, based on the fact that both of these organ sources (cervix & breast) were from females, we speculated that there might be a high specificity in controlling AGE-R and Ubc9 complex signaling in cancer cells depending on the chemoresistance of anti-cancer drugs in breast cancer. This is important for SURMylation and nucleolar delocalization of topoisomerase (topo) I in response to topo I inhibitors such as topotecan[19]. These results suggest Ubc9 might be involved in controlling chemoresistance via AGE-R in cervical cancer cells, but not in breast cancer cells. It remains to be seen whether the mechanism is differentially regulated in two different cancer systems: cervical and breast cancer.

Co-localization of AGE-R and UBP in mouse aortic vascular atherosclerotic plaque

Ubc9 was previously shown to directly interact with SURM-1 (Ubc9 binding protein: UBP)[20-23]. Ubc9, SURM-1, and AGE-R complex are not limited to cancer cells, but may represent a

more ubiquitous level of control of AGE-R function. Previous studies suggest that the ligand of AGE-R, S100b, enriched in atherosclerotic plaques and expanding neointima after acute arterial injury, together with AGEs, amplify proinflammatory mechanisms in the vessel wall, especially in diabetes[1,24]. We speculated that expression patterns of AGE-R and UBP might be correlated in mouse atherosclerosis model. To determine the relevance of AGE-R and UBP in type 1 diabetic vascular athsclerotic lesions, we utilized the confocal laser microscopy on the atherosclerotic plaques of *ApoE*-null mice rendered diabetic with streptozotocin[1] stained with anti-AGE-R antibodies and anti-SURM1 as shown in Figure 3.

An atherosclerotic plaque lesion tissue section was analyzed with anti-AGE-R antibodies (solid green: Figure 3A) and anti-SURM1 (UBP) antibodies (solid red: Figure 3B) with confocal laser microscopy. The colocalization of AGE-R antibodies and SURM1 (UBP) antibodies were detected at the neointima area in atherosclerotic plaque lesion (yellow overlapped region: Figure 3C), known to be abundant in various types of cells including monocytes, macrophages, endothelial cells and smooth muscle cells. While some cells were specific to AGE-R antibodies only (solid green: Figure 3C), other cells only had SURM-1 antibodies (solid red: Figure 3C). The plaque was stained with anti-alpha Actin antibodies or anti-CD68 antibodies indicating the presence of SMC (smooth muscle cells) and monocytes/macrophages, respectively (data not shown). These results strongly suggested that interaction of AGE-R and SURM-1 (UBP) is cell-type specific to atherosclerotic plaque lesion. These findings suggest that AGE-R is associated with UBP and Ubc9 complex in vascular atherosclerotic lesions in the diabetic model. These results are important parallels to the potential impact of vascular AGE-R and Ubc9 signaling; a key property of AGE-R in tumor cells is its ability to mediate cellular migration[12].

In conclusion, we have investigated whether the expression and distribution of Ubc9 and AGE-R are closely associated with diabetic antherosclerosis and cancer in mice. We have demonstrated the importance of the PEST (E_{371}-E_{398}) domain in mediating interactions with both Ubc-9 and SURM-1.

CONCLUSION AND PROSPECTS

Further studies are needed to characterize how specific cell types differently interact with AGE-R and/or UBP to understand the underlying molecular mechanism of its physiological function. We have shown that AGE-R and Ubc-9 proteins are coexpressed in atherosclerotic plaque in Figure 3. To further confirm this expression pattern, we used the previous findings that Ubc-9 is known to bind to SURM-1[20-23]. Ubc9, SURM-1 (Ubc9 binding protein: UBP) and AGE-R complexes are not limited to cancer cells, but may represent a more ubiquitous level of control of AGE-R function. We have tested whether AGE-R would also be coexpressed with SURM-1 in plaque. The confocal microscopy study indicated that AGE-R would also be colocalized with SURM-1 in mouse aortic root athersclerotic plaque.

Our results suggest that Ubc9 is important in mediating AGE-R signaling modulation through direct or indirect interactions with SURM-1 modification. Although the physiological implications of SURMylation still need to be explored further, this study provides a solid basis for AGE-R and SURM signaling cascade.
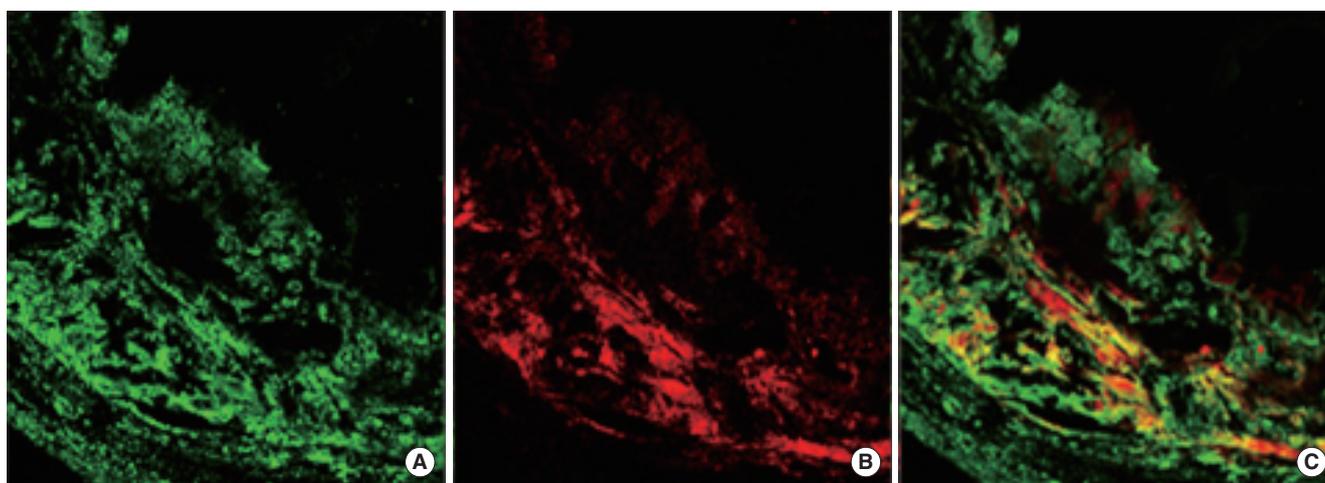

Figure 3. Colocalization of AGE-R and SURM-1 proteins (Ubc9 binding protein: UBP) in Mouse Aortic root blood vessel Atherosclerotic plaque. The aortic root atherosclerotic plaque in *ApoE*^{-/-} mice rendered diabetic with streptozotocin was stained with anti-AGE-R antibodies and anti-SURM-1(UBP). Atherosclerotic plaque lesion tissue section was analyzed with anti-AGE-R (green: A) and anti-SURM1 (UBP) (red: B) antibodies with the confocal laser microscopy. The colocalization of AGE-R antibodies and SURM1 (UBP) antibodies were detected at the neointima area in atherosclerotic plaque lesion (area in yellow: C), known to be abundant of various types of cells including monocytes, macrophages, endothelial cells and smooth muscle cells. However, some cells are specific to AGE-R antibodies only (Green color only: C), other cells are only with SURM-1 antibodies (Red color only: C).

MATERIALS AND METHODS

Animals

Hyperlipidimic Apolipoprotein $ApoE^{-/-}$ mice (C57BL/6J background strain, male) were purchased from Charles River Laboratories (Wilmington, MA), and administered with streptozotocin (STZ) for 3 weeks to induce diabetes as a model of *in vivo* diabetic atherosclerosis[1,25-27]. $Apoe^{-/-}$ mice were weaned at 4 weeks of age onto a high-cholesterol diet for 12 weeks[1,26,27]. Littermate $Apoe^{+/+}$ mice were used as controls. The mice were perfused with 4% paraformaldehyde and then aortic root vascular vessels were isolated to make the frozen blocks with Tissue-Tek OCT 4583 compound medium solution (http://www.diacomp.org; Animal Models of Diabetic Complications Consortium 2003)[1,26,27].

Computer database search

The Sequence Manipulation Suite (SMS), a computer search program, was employed to measure the alignment score between AGE-R and E2A protein sequences (http://www.bioinformatics.org). BLOSUM62 was used as a scoring matrix. The value for gaps preceding a sequence was 0. The value for internal gaps was -2. The value for gaps following a sequence was 0. (http://www.bioinformatics.org).

Cells

Human cervical adenocarcinoma epithelial cells (HeLa), human breast adenocarcinoma epithelial cells (MDA-MB-231), Rat C6 glioma cells and Chinese Hamster Ovarian (CHO) cells were purchased from ATCC (Manassas, VA). AGE-R overexpressing CHO cells were stably transfected with full length AGE-R wild type cDNA (A. Schmidt, New York, NY) and selected for a stable clone[1]. The cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen GIBCO BRL, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen GIBCO BRL, Carlsbad, CA) in the presence of 5% CO_2. MDA-MB-231, LM2-4142, and MDA-MB-468 cells were grown in RPMI 1640 (Cambrex, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS)[28].

Chemicals & antibodies

S100b protein was purchased from Calbiochem (La Jolla, CA). Streptozotocin and paraformaldehyde were purchased from Sigma (St. Louis, MO). Tissue-Tek OCT 4583 compound medium solution was purchased from Sakura Finetec (Torrance, CA). Rabbit, goat, and mouse anti-AGE-R antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies against SURM-1 (SUMO1) were purchased from Invitrogen Zymed (San Francisco, CA). Goat polyclonal antibodies against Ubc9 were purchased from Abcam (Cambridge, MA) and Rabbit polyclonal antibodies against

Ubc9 were purchased from Invitrogen Zymed (San Francisco, CA). Texas Red-coupled anti-mouse antibodies were purchased from Amersham and FITC-coupled anti-rabbit antibodies were purchased from Dako. Vectorshield mounting media were purchased from Vector Labs (Burlingame, CA)[28].

Confocal laser microscopy

ApoE-null mice aortic root vessel atherosclerotic plaque section slides were stained with both anti-Ubc9 and anti-AGE-R antibodies. Controls for nonspecific immunostaining were performed in the absence of primary Abs. No immunostaining was detected in negative controls (no primary Abs). Slides were mounted with Vectorshield mounting media and analyzed with an oil immersion objective using a Nikon E800 microscope. Images were made with the Bio-Rad Radiance 2000 Confocal System and Lasersharp 2000 software (Bio-Rad, Hercules, CA)[29]. Cells were fixed in methanol at room temperature for 5 min and blocked with 1X PBS containing 10% fetal calf serum and 0.1% Tween 20. In a single-labeling experiment, AGE-R was detected with mouse monoclonal antibodies against AGE-R. Ubc-9 protein was detected with rabbit polyclonal antibodies against Ubc9. For a double labeling experiment, AGE-R was detected with the mouse monoclonal antibodies directed against AGE-R and Ubc9 was detected with the rabbit anti-Ubc9 antibody. Primary antibodies were diluted in block solution and incubated with the permeabilized cells for 1 hr at room temperature. Secondary antibodies such as Texas Red-coupled anti-mouse antibodies and FITC-coupled anti-rabbit antibodies were incubated with cells for 30 min at room temperature[29]. The membranes were washed three times in 1X PBS followed by a final wash in 1X PBS containing 0.05% Tween for 15 min. Preparations were examined by confocal laser scanning microscopy using an MRC 1000 inverted confocal microscope (Bio-Rad) or an inverted Diaphot 300 microscope (Nikon)[29]. Images were collected using an oil immersion lens and using excitation wavelengths of 543 nm (for Texas Red) and 488 nm (for FITC)[29].

Western blotting

Cells were lysed with 1% triton X-100 buffer and boiled for 5 min in SDS-sample buffer (Invitrogen). Cell lysates were then separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham). These membranes were blocked with 1% fat-free milk in 1X PBS, and then incubated with the polyclonal antibodies in blocking solution for 1 hr at room temperature. Subsequently, membranes were incubated with peroxidase-conjugated secondary antibody in block solution (Dako). Each of the incubation steps were followed by 3 washes for 15 min in PBS containing 0.1% Tween 20. Development was performed as described in the ECL protocol (Amersham).

ACKNOWLEDGEMENTS

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