Characterization of the functional domains and quantification in the cells of a steroid receptor-binding protein, SRB-RGS

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ABSTRACT We isolated the cDNA of a steroid receptor-binding protein, SRB-RGS, binding to the estrogen receptor (ER)α. SRB-RGS very efficiently suppressed the ERs-mediated transcriptional activities. SRB-RGS interacted with ER on DNA. SRB-RGS was localized in both the nucleus and cytoplasm in the cells. SRB-RGS induced cell death of HeLa cells. In the present paper, we examined the functional domains of SRB-RGS. The effects of SRB-RGS on the ERs-mediated transcriptional activities were exerted by the PDZ domain-containing region and/or the RGS domain-containing region of SRB-RGS. The nuclear localization signal (NLS) was in the RGS domain containing one basic region. Either the PDZ or RGS domains on SRB-RGS induced cell death. Either the PDZ or RGS domains on SRB-RGS were necessary for transcriptional suppression, subcellular localization and induction of the death in HeLa cells. The expression of SRB-RGS was observed in the cytoplasm or both the cytoplasm and nucleus of various types of cells by confocal laser-scanning microscopy after immunostaining of the cells. SRB-RGS was expressed in all types of cells examined by using real-time RT-PCR.

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Key words：**SRB-RGS/RGS3, Estrogen receptor, Functional domain, Quantification**

INTRODUCTION

The biological actions of estrogens are mediated through binding to two specific estrogen receptors (ERs), ERα or ERβ, which belong to the nuclear receptor superfamily, a family of ligand-regulated transcriptional factors. Steroid hormone receptors have been indicated to be composed of six major functional domains, *i.e.*, the A/B domains in the NH_2-terminal region of the protein as the activation function 1 (AF-1), domain C as the DNA-binding domain arranged in two zinc-stabilized DNA-binding motifs, domain D as a hinge region which contains a nuclear localization signal, and domain E/F as the ligand-dependent transcription domain (activation function 2 (AF-2)). For the activation of transcription, the steroid hormone receptors recruit

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coactivators allowing the local decondensation of chromatin. In the next step, the DRIP/TRAP complex binds to the AF-2 of the receptor and mediates transcriptional activation. The receptors in the absence of ligands bind to the corepressors, such as N-CoR and SMRT. The corepressors recruit histone deacetylase (HDAC) and thereby suppress the transcriptional activity[1-4].

G-protein coupled receptors (GPCRs) on the cell surface mediate activation of effectors in many physiological responses. Agonist binding to the GPCR induces the direct activation of the Gα subunit, triggering the exchange of guanosine-5'-diphosphate (GDP) to guanosine-5'-triphosphate (GTP), and G$\beta\gamma$ is capable of activating or inhibiting effectors. The intrinsic GTPase activity of Gα is quite slow. The regulator of the G-protein signaling (RGS) protein for Gα subunits enhances the hydrolysis of GTP bound to Gα. This GTPase-activating protein (GAP) activity enhances G-protein deactivation[5-9].

We have cloned the cDNA of the protein that interacted with domains C and D of the rat-origin estrogen receptor α (rERα C/D) from a rat ovary cDNA library, which was the cDNA of a novel steroid receptor-binding (SRB) protein bearing a PDZ domain at the NH_2-terminal and the regulator of the G-protein signaling (RGS) domain at the COOH-terminal, designated as SRB-RGS. SRB-RGS very efficiently suppressed the ERs-mediated transcriptional activities. The interactions between SRB-RGS and ERs were revealed by a coimmunoprecipitation assay and a mammalian two-hybrid system. A gel shift assay revealed that SRB-RGS interacted with ER on the DNA. Green fluorescence of enhanced green fluorescence protein (EGFP)-tagged SRB-RGS was observed in both the nucleus and the cytoplasm in the cells by confocal laser-scanning microscopy. Overexpression of SRB-RGS induced cell death of HeLa cells. Intrinsic SRB-RGS was observed by Western blotting and by confocal laser-scanning microscopy after immunostaining of the cells. SRB-RGS was recently registered as G-protein signaling 3 (RGS3) in the secondary DNA database (Accession No.NM019340) of the National Center for Biotechnology Information (N.C.B.I.) of the U.S. National Library of Medicine (Bethesda MD, U.S.A.) by others. Six kinds of human-origin (h)RGS3 are known (Fig.1). SRB-RGS is called isoform 1 of rat RGS3 on Entrez Gene of N.C.B.I. The other rat isomers are not known yet. SRB-RGS

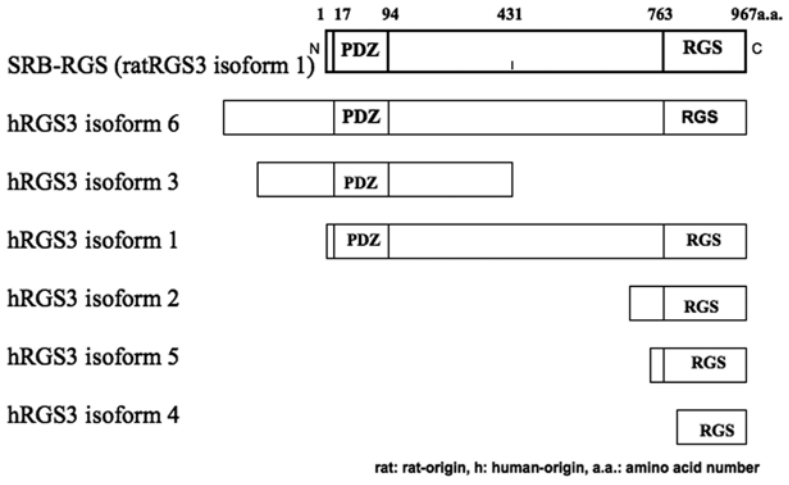


Fig. 1. A schematic representation of the SRB-RGS and human-origin (h)RGS3 isoforms. The amino acid (aa) numbers of SRB-RGS are shown above the schema. PDZ and RGS represent the PDZ and RGS domains, respectively.

is the first RGS3 for which the full-length cDNA has been registered (DDBJ/GenBank/EMBL Accession No.AB055153)[2, 3].

The amino acids (aa) (SRB-RGS aa839-899 and aa909-954) for the alpha-helical structure (α1 to α5 and α6 to α9, respectively) of the RGS domains on SRB-RGS are conserved among species. SRB-RGS asparagine-908 (N) corresponding to the critical asparagine-128 of RGS4 to interact with the Gα subunit and cause GTPase acceleration is also conserved. The regions aa789-792 and aa 808-810 of SRB-RGS (two Bs in the RGS domain) correspond to two stretches of the positively charged amino acids of partial RGS3, Arg^{341}LysArgLys and Arg^{360}ArgArg, which have been reported as the putative nuclear localization signal (NLS) sequence. Overexpression of a shorter form of the partial hRGS3 (called hRGS3T) followed by serum deprivation induced apoptosis of CHO cells. The acidic region (SRB-RGS aa651-675) was presumed to be a coiled coil[5, 6]. The PDZ domain is a multi-functional protein-protein interaction module that plays important roles in organizing signal transduction complexes, clustering membrane proteins and maintaining cell polarity[7, 10, 11].

In this paper, the effects of SRB-RGS on suppression of the ERs-mediated transcriptional activities were exerted by the PDZ domain-containing region and the RGS-containing region of SRB-RGS using deletion mutants of SRB-RGS. The two regions may act on the suppression of the ERα- and ERβ-mediated transcriptional activities. The nuclear localization signal (NLS) was in the RGS domain containing at least one basic region. Either the PDZ or RGS domains on SRB-RGS induced cell death. The expression of SRB-RGS was observed by confocal laser-scanning microscopy. SRB-RGS was found to be expressed in all the cell types examined by quantitative real-time RT-PCR.

MATERIALS AND METHODS

Construction of plasmids

The full-length SRB-RGS expression vector, pcDNASRB-RGS aa1-967 (Fig.2 No.10), was constructed by inserting SRB-RGS cDNA into the multi-cloning site (MCS) of pcDNA3 (Fig.2 No.1) (Invitrogen Co., Groningen, The Netherlands) driven by a cytomegalovirus (CMV) promoter as described previously[2, 3]. The expression vector of the deletion mutants of SRB-RGS was constructed by modifying pcDNASRB-RGS. The construction of the expression vectors of hERα, pcDNA-hERα and hERβ, pcDNA-hERβ was described previously[2, 3].

The expression vector for the EGFP-tagged full-length SRB-RGS aa1-967, pEGFPSRB-RGS aa1-967, was constructed by inserting the cDNA fragment of SRB-RGS PCR-amplified from the expression vector, pcDNASRB-RGS into the EcoRI site of MCS of pEGFP-C1 (Clontech Co., Palo Alto, CA, U.S.A.). The expression vectors of the EGFP-tagged deletion mutants of SRB-RGS were constructed by modifying pEGFPSRB-RGS aa1-967.

Cells, cell culture and transfection

COS-7 cells (an African green monkey kidney fibroblast cell line), HeLa cells (a human epithelial cervical cancer cell line), PC-12D cells (a rat pheochromocytoma cell line), rat-1 cells (a rat fibroblast-like cell line) and HUC-F2 cells (a normal umbilical cord fibroblast, female) were purchased from The Institute of Physical and Chemical Research (RIKEN) (Wako, Japan). MCF-7 cells (a human breast cancer cell line) was from the Health Science Research Resources Bank (Osaka, Japan). A431 cells (a human epidermoid carcinoma cell line) were a gift from Dr. Okamoto (Kawasaki Medical School). MDA-MB-231 cells, MDA-MB-157 cells, KPL-1 cells, KPL-4 cells, KPL-3C cells, BT-474 cells and HCC1937 cells (human breast cancer cell lines) are maintained

by Dr. Kurebayashi (one of the authors). The cells were routinely maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Sigma Co., St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (FBS) (Medical & Biological Laboratories Co., Nagoya, Japan) in 5% CO_2 at 37℃. The cells grown in screw-capped flasks (25 cm^2) or on glass cover slips in 35-mm dishes were transiently transfected with plasmids by lipofectamine2000 (Invitrogen Co., CA, U.S.A.) and incubated for 48 h after transfection.

Chloramphenicol acetyltransferase assay

The chloramphenicol acetyltransferase (CAT) assay, using a FAST CAT Yellow (deoxy) CAT assay kit (Molecular Probes, Eugene, OR, U.S.A.), and the β-galactosidase (β-gal) assay were performed as described previously[3]. Briefly, the medium for COS-7 cells in a screw-capped flask (25 cm^2) was replaced by phenol red-free MEM (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% dextran-coated charcoal-treated (DCC) FBS for 48 h before transfection. The COS-7 cells were cotransfected with plasmids as indicated and incubated for 48 h in the presence of 10^{-7} M estradiol (E_2) after transfection. The cell extract was prepared according to the manufacturer's manual. A portion of the cell extract was incubated with fluorescent deoxychloramphenicol substrate and acetyl CoA at 37℃ for 2 h. The acetylated derivative and the substrate were quantified by measuring the absorbance using a Model F-3010 fluorescence spectrophotometer (Hitachi Co., Tokyo, Japan). For the β-gal assay, the other portion of the cell extract was incubated with o-nitrophenyl β-galactopyranoside at 37℃ for 30 min. The relative CAT activities were calculated according to the manufacturer's manual and normalized by β-gal activities. The CAT activity for the cells cotransfected with the empty plasmid, pcDNA3 instead of pcDNA-hERα or pcDNA-hERβ was subtracted from each CAT activity.

Anti-SRB-RGS antibody

The anti-SRB-RGS peptide antiserum was prepared as mentioned previously[2]. The anti-SRB-RGS peptide antiserum was raised against a 18-amino acid peptide sequence corresponding to SRB-RGS residues 504-521 (QEA APG PES PSS EDI ATC). The peptide was conjugated to keyhole lympet hemocyanin (KLM) as a carrier with SH group of COOH-terminal cysteine and immunized a rabbit. The immune and preimmune sera were purified and called as the anti-SRB-RGS antibody and the preimmune immunoglobulin (IgG), respectively.

Immunostaining of the cells

The cells grown on glass cover slips were fixed in 4% paraformaldehyde for 1 h at room temperature. After permiabilization with 2% Triton X-100 in PBS for 10 min at room temperature, the cells were blocked with 20% FBS in PBS and incubated with the anti-SRB-RGS antibody or preimmune serum and then fluorescein-5-isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin (IgG, IgA and IgM) (ICN Pharmaceuticals, Aurora OH, U.S.A.). They were observed on a confocal laser-scanning microscopy as described below.

Observation of subcellular localization and TUNEL assay

The HeLa cells or COS-7 cells grown on glass cover slips in 35-mm dishes were transiently transfected with pEGFP-C1, pEGFPSRB-RGS or pEGFPSRB-RGS derivatives (2μg for observation of subcellular localization, 3μg for TUNEL assay). The TUNEL assay was performed as mentioned previously[3]. Briefly, an ApoTag^R Red *In Situ* Apoptosis Detection kit (Intergen Co., NY, U.S.A.) was used for the assessment of DNA fragmentation in the cells transfected with the plasmids after

fixation of the cells on glass cover slips. DNA fragments were labeled with the digoxigenin-nucleotide and then bound an anti-digoxigenin antibody conjugated to Rhodamine. The cells were viewed on a confocal laser-scanning microscope as mentioned below.

Observation of the cells by confocal laser-scanning microscopy

The cells in 35-mm dishes were grown on glass cover slips in DMEM supplemented with 10% FBS[2, 3]. The cells were washed with PBS and fixed in 4% paraformaldehyde for 1 h. The cover slips were mounted on glass slides after or without propidium iodide (PI) staining of the cells. The cells were viewed on a Fluoview FV300 confocal laser-scanning microscope (Olympus Co., Tokyo, Japan).

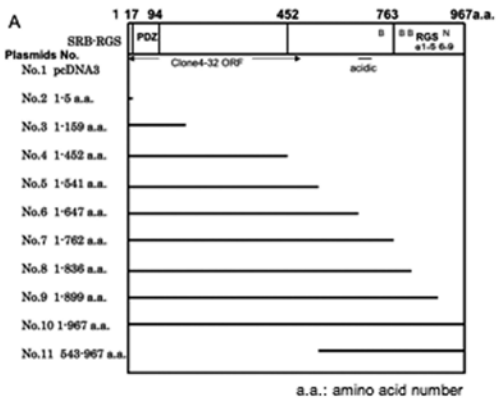
Real-time RT-PCR

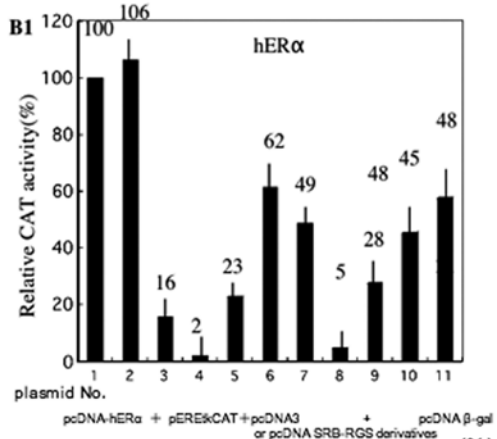
Total RNA was prepared from the cells by using an RNeasy PLUS Mini kit (Quiagen GmbH, Hilden, Germany) according to the manufacturer's manual. Briefly, the homogenized lysate was centrifuged in a DNA Eliminator spin column and in an RNeasy spin column to get total RNA. The RNA extracts were incubated with RNase-free DNase (Promega Co., Madison, WI, U.S.A.). The total RNA was converted to cDNA by iScript cDNA synthesis kit (Bio-Rad Co., Hercules, CA, U.S.A.). Control cDNAs were prepared by no-reverse transcriptase reactions in which reverse transcriptase was omitted from the reverse transcriptase reaction. All values were subtracted the values from control cDNAs. Power SYBR green-based real-time RT-PCR Master Mix (Applied Biosystems, Warrington, U.K.) using a 7300 Fast Real-Time PCR System or PRISM^{R} 7700 Sequence Detection System (Applied Biosystems). The following primer pairs were used with PRISM 7700 Sequence Detection System: 5'-ATC AGG ATA TGT GGC GGA TGA-3' (forward) and 5'-CTG ATT TGT GTA GTC GGT TTA TGC A-3' (reverse) for amplification of β-gal; 5'-CCA CCA ACC AGT GCA CCA TT-3' (forward) and 5'-GGT CTT TTC GTA TCC CAC CTT TC-3' (reverse) for amplification of hERα; and: 5'-AGA GTC CCT GGT GTG AAG CAA G-3' (forward) and 5'-GAC AGC GCA GAA GTG AGC ATC-3' (reverse) for amplification of hERβ. The plasmids pcDNAβ-gal, pcDNA-hERα and pcDNA-hERβ were used as standards for the quantification for β-gal, hERα and hERβ cDNAs, respectively. The following primer pairs were used in conjunction with the 7300 Fast Real-Time PCR System: 5'-ATT GGA GCT CGC AGC TCA GA-3' (forward) and 5'-GCT TCT CAC AAG GCA TTG GC-3'(reverse) (these primers amplify a part of the STS sequenced directly from African green monkey genomic DNA with the human forward and reverse primers) for amplification of SRB-RGS; and 5'-GTA ACC CGT TGA ACC CCA TT-3' (forward) and 5'-CCA TCC AAT CGG TAG TAG CG-3' (reverse) for amplification of human 18S ribosomal (r)RNA as an endogenous control[12]; as well as the above primer pairs for amplification of hERα and hERβ. The values were normalized to the respective value for 18S rRNA. The reactions were performed at 95℃ for 10 min followed by 40 cycles of 95 ℃ for 15 sec and 60℃ for 1 min.

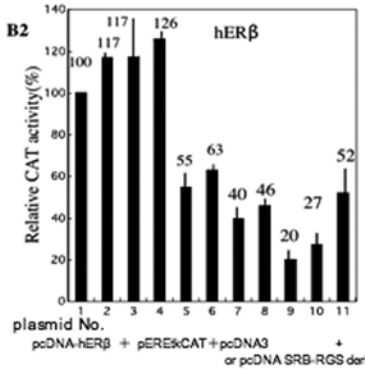
RESULTS

Functional domain of SRB-RGS for the transcriptional suppression

SRB-RGS very efficiently suppressed the ERs-mediated transcriptional activities[2, 3]. SRB-RGS holds two major putative functional domains, PDZ and RGS domains. We examined the functional domain(s) of SRB-RGS involved in suppression of the hERα- and hERβ-mediated transcriptional activities. As indicated at the bottoms of Fig.2 B1 and 2 B2, the expression vectors of hER α, pcDNA-hERα or hERβ, pcDNA-hERβ (or their empty plasmid, pcDNA3) and the reporter







B3 (%)

plasmid No.	1	2	3	4	5	6
hERα/β-gal (B1)	100	106±24	120±25	89±8	112±8	109±11
hERβ/β-gal (B2)	100	92±15	102±9	158±9	134±37	114±33

plasmid No.	7	8	9	10	11
hERα/β-gal (B1)	94±11	89±29	108±11	107±14	91±20
hERβ/β-gal (B2)	102±19	108±13	116±20	125±31	127±13

B4

intrinsic SRB-RGS/18S rRNA : 1545 ± 509
overexpressed hERα/18S rRNA: 12767 ± 3485

r: ribosomal

Fig. 2. Functional domain of SRB-RGS for the transcriptional suppression. (A) Functional domains and derivatives of SRB-RGS. PDZ domain: a protein/protein interaction module; RGS domain: the regulator of G-protein signaling domain. (B1 and 2) Effects of SRB-RGS derivatives on ERα- or ERβ-mediated transcription activities. COS-7 cells were transiently transfected with plasmids as indicated at the bottom of the figure. The cells were incubated for 48 h in the presence of 10^{-7} M estradiol. The CAT activity for the cells cotransfected with the empty plasmid, pcDNA3 instead of pcDNA-hERα or pcDNA-hERβ, was subtracted from each CAT activity. In each graph, the activity in cells transfected with pcDNA3 (No.1) was represented as 100%. The numbers at the top of the bars in the graphs represent the percent activity. The numbers under the x-axes represent the plasmid numbers shown above. Values are the means of three or more independent experiments±SD. (B3) Quantitative analysis of the estrogen receptors. The amount of hERα or hERβ overexpressed in COS-7 cells in the above experiments (B1 and B2) was quantified by real-time RT-PCR. The values were normalized by the respective β-gal value. The values are the means of three or more independent experiments±SD. (B4) Quantitative analysis of intrinsic SRB-RGS and overexpressed hERα The amounts of intrinsic SRB-RGS and overexpressed ERα in COS-7 cells in the above experiment (B2 No.1) were quantified by real time RT-PCR. The values were normalized by the respective value of 18S ribosomal (r)RNA value. The values are the means of three or more independent experiments. The values by plasmid No.1 were represented as 100%.

plasmid for ERs-mediated transcriptional activities, pEREtkCAT, were also cotransfected into COS-7 cells along with the expression vector of the SRB-RGS derivatives (the full length or the deletion mutants of SRB-RGS), the pcDNASRB-RGS derivatives or the empty plasmid, pcDNA3. The expression vector of β-gal, pcDNAβ-gal, was cotransfected to normalize the transfection efficiency. The full-length SRB-RGS (No.10) effectively suppressed the hERα- and hERβ -mediated transcriptional activities (Fig.2 B1 and 2 B2; Nos.10, respectively), as described previously[3]. Stepwise deletion of the RGS domain-containing region (Nos.3-9 for hERα, Nos.5-9 for hERβ) did not release the activity of SRB-RGS. Deletion of SRB-RGS aa6-159 from SRB-RGS aa1-159 or

SRB-RGS aa6-541 from SRB-RGS aa1-541 released the transcriptional suppression activities on the hERα- and hERβ-mediated transcriptional activities (Fig.2 B1 and 2 B2; Nos.2, respectively). Both SRB-RGSs aa6-159 and aa6-541 bore the PDZ domains. The N-terminus-deleted RGS domain-containing region (with effective regions, aa6-159, aa6-541 deleted) (No.11) was still effective for the suppression of the hERα- and hERβ-mediated transcriptional activities (Fig.2 B1 and 2 B2; Nos.11, respectively).

The amounts of hERα and hERβ expressed in Fig.2 B1 and 2 B2, respectively, were quantified by real-time RT-PCR (Fig.2 B3). The amounts of hERα and hERβ expressed were normalized the transfection efficiency by the amount of β-gal expressed. The relative amounts among hERα expressed were similar each other when compared to the values of No.1 represented as 100 % (Fig.2 B3 hERα/β-gal No.1-11). Also the relative amounts among hERβ expressed were similar each other (Fig.2 B3 hERβ/β-gal No.1-11). That is, the differences among the CAT activities in Fig.2 B1 and 2 B2 were not caused by the differences of the amount of the hERα and hERβ expressed.

As mentioned below, the COS-7 cells express intrinsic SRB-RGS. We quantified the levels of intrinsic SRB-RGS and hERα overexpression under the condition in Fig.2 B1 No.1 by real-time PCR. The amount of intrinsic SRB-RGS was about 12% of the amount of hERα overexpressed (Fig.2 B4). In these experiments, the influence of intrinsic SRB-RGS was likely not pronounced. And we could compensate for this effect by the use of the controls (Fig.2 B1 and 2 B2 Nos.1)

Functional domain of SRB-RGS for subcellular localization

We previously demonstrated that the EGFP-tagged SRB-RGS was localized in both cytoplasm and nucleus of COS-7 cells[3]. Here, we examined the functional domain of SRB-RGS for subcellular localization. The expression vectors of the EGFP-tagged SRB-RGS derivatives (Fig.3) were transfected into COS-7 cells. The EGFP itself, as well as the EGFP-tagged SRB-RGSs aa1-103 and aa1-543, were localized in both the cytoplasm and nucleus. They

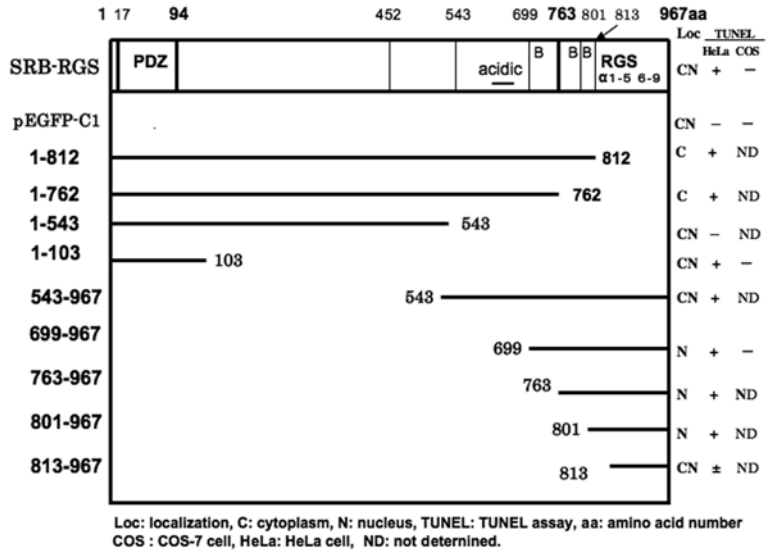


Fig. 3. A schematic representation of EGFP-tagged SRB-RGS and SRB-RGS derivatives. The numbers are the amino acid (aa) numbers. On the right side of the figure, the results (subcellular localization and TUNEL assay in Figs.4 and 5) obtained using these plasmids are summarized. B: the positively charged amino acids;

do not have the remarkable nuclear localization abilities. There are three regions of basic amino acids on SRB-RGS, aa708-712, aa789-794, aa808-810, which are indicated with the letter B in Figs.2 and 3. These regions were candidates for the nuclear localization signals. We examined whether these regions had nuclear localization ability. The EGFP-tagged SRB-RGSs aa1-812 and aa1-762 were localized in the cytoplasm. SRB-RGS aa813-967 likely has the nuclear localization signal(s). We examined whether the COOH-terminal domain itself had nuclear localization ability. The EGFP-tagged SRB-RGSs aa699-967, aa763-967 and aa801-967 bore three, two and one basic region(s), respectively, and were all localized in the nucleus. SRB-RGS aa813-967 (with the three basic regions deleted) reduced the nuclear localization ability and was localized in both the cytoplasm and nucleus. And as mentioned above, SRB-RGS aa1-812, in which three basic regions were cotained, was localized in the cytoplasm. Consequently, SRB-RGS aa813-967 was a least region containing the nuclear localization ability. The nuclear localization ability of SRB-RGS aa813-967 was enhanced by the addition of at least one basic region (see SRB-RGS aa801-967). EGFP-tagged SRB-RGS aa543-967 (with an acidic region contained) was localized in both the cytoplasm and nucleus. The acidic region-containing region reduced the nuclear localization ability of SRB-RGS aa699-967.

Functional domain of SRB-RGS for inducing cell death

Overexpression of SRB-RGS in HeLa cells induced cell death[3]. That is, HeLa cells which the green fluorescence of the overexpressed EGFP-tagged SRB-RGS was observed were TUNEL-positive (*i.e.* that showed red fluorescence). Here, we examined the functional domain of SRB-RGS that was responsible for the induction of cell death. The expression vectors of EGFP itself or the EGFP-tagged SRB-RGS derivatives shown in Fig.3 were transfected

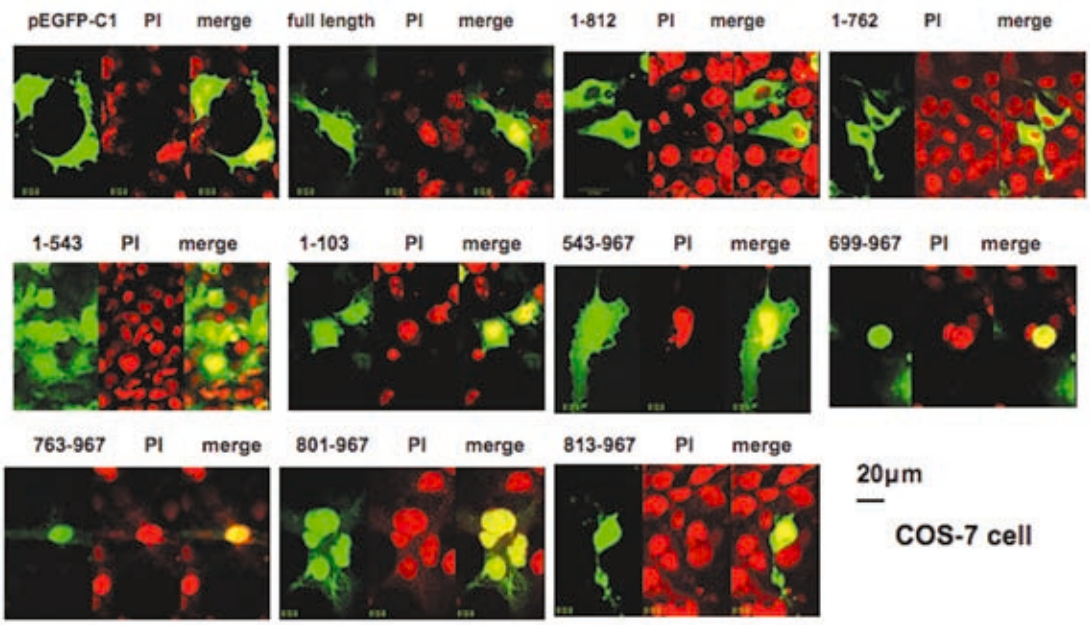


Fig. 4. Functional domain for subcellular localization. The COS-7 cells were transiently transfected with pEGFP-C1, pEGFPSRB-RGS or pEGFPSRB-RGS derivatives. Fluorescence was observed by a confocal laser-scanning microscope after staining of the nucleus with PI. Green fluorescence: EGFP; red fluorescence: PI.

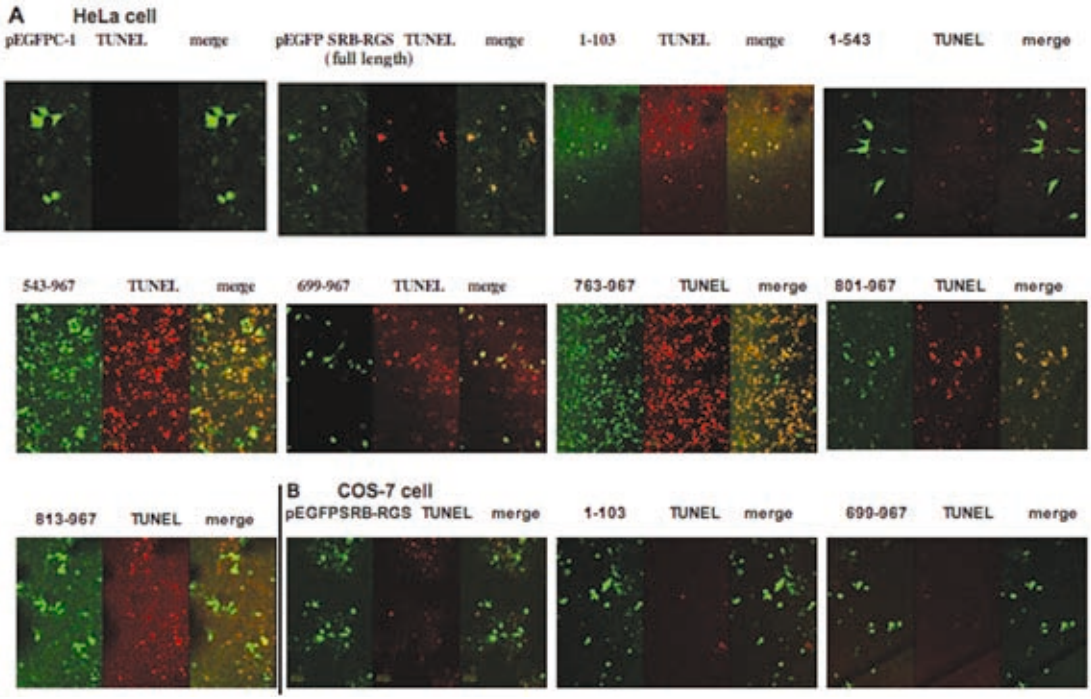


Fig. 5. Functional domains for cell death. The HeLa cells (A) or COS-7 cells (B) grown on glass cover slips in 35-mm dishes were transiently transfected with pEGFP-C1, pEGFPSRB-RGS or pEGFPSRB-RGS derivatives (3μg each). Fluorescence was observed by a confocal laser-scanning microscope after the TUNEL assay was performed on the cells to assess DNA fragmentation, as described in the **MATERIALS AND METHODS**. Red fluorescence: staining by TUNEL assay; green fluorescence: EGFP.

into HeLa cells or COS-7 cells. Fluorescence was observed by confocal laser-scanning microscopy after the TUNEL assay was performed on the cells to assess DNA fragmentation. EGFP itself did not induce cell death of HeLa cells. The EGFP-tagged full-length SRB-RGS (green fluorescence) induced cell death (red fluorescence) as shown by the color yellow (merge) (Fig.5). Although SRB-RGS aa1-103 induced cell death, SRB-RGS aa1-543 did not induce. The cell death-inducing ability of SRB-RGS aa1-103 may be inhibited by SRB-RGS aa104-543. SRB-RGSs aa1-762 and aa1-812 (with SRB-RGS aa1-543 plus aa544-762 contained and RGS domain deleted) induced cell death. The inhibition (by SRB-RGS aa103-543) of the cell death-inducing ability of SRB-RGS aa1-103 was cancelled by aa543-762. Next, we examined whether the RGS domain is necessary for cell death. The SRB-RGSs aa543-967, aa699-967, aa763-967 (with the whole RGS domain contained) and aa801-967 induced cell death. The SRB-RGS aa813-967 almost did not induce cell death. The SRB-RGS aa801-967 was a COOH-terminal region required for cell death. Taken together, either the PDZ domain (SRB-RGS aa1-103) or the RGS domain (SRB-RGS aa801-967) was the cell death-inducing domain.

On the other hand, the EGFP-tagged full-length SRB-RGS and SRB-RGSs aa1-103 and aa699-967 as well as EGFP itself[3] did not induce cell death of COS-7 cells (Fig.5B).

Examination of SRB-RGS expression by confocal laser-scanning microscopy and by quantitative real-time PCR

SRB-RGS suppressed the ERs-mediated transcriptional activities as described previously[2, 3].

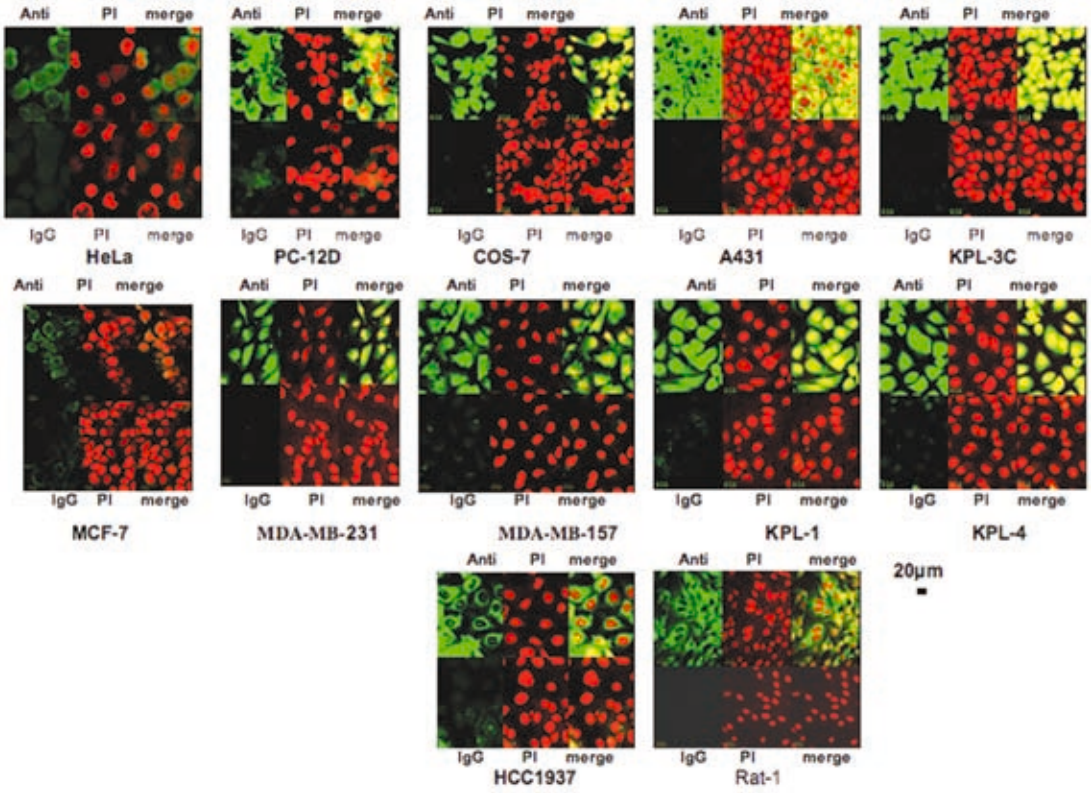


Fig. 6. Immunostaining of the cells. The cells were stained with the anti-SRB-RGS antibody (Anti) or preimmune rabbit immunoglobulin (IgG) and then fluorescein-5-isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin (green fluorescence). They were viewed on a confocal laser-scanning microscope after PI staining of the nucleus (red fluorescence).

A reduced amount and/or functional defect of SRB-RGS may affect the cell growth *via* release of the suppression. We examined the SRB-RGS expression in the cells by confocal laser-scanning microscopy and by quantitative real-time RT-PCR.

The expression of SRB-RGS was observed by confocal laser-scanning microscopy after immunostaining of the cells. SRB-RGS was observed in both the cytoplasm and nucleus or in the cytoplasm in all types of cells examined by immunostaining (Fig.6). In HeLa, PC-12D, A431, rat-1 cells, HCC1937 and MCF-7 cells, SRB-RGS was observed in the cytoplasm or in both the cytoplasm and nucleus. In COS-7, KPL-3C, MDA-MB-231, MDA-MB-157, KPL-1 and KPL-4 cells, SRB-RGS was localized in both the cytoplasm and nucleus.

The SRB-RBS expressed in breast cancer cells and the other types of cells was quantified by real-time RT-PCR. Similar level of SRB-RGS was expressed in all the types of cells examined irrespective of the presence or absence of ERs and the target (MCF-7, MDA-MB-231, KPL-1, KPL-4, KPL-3C, BT-474, MDA-MB-157, HCC1937 and HeLa cells)[13] or non-target cells (A431 cell, HUC-F2, COS-7 cells) for estrogen with exception of the expressed level in COS-7 cells (Fig.7).

DISCUSSION

We isolated the cDNA of a steroid receptor-binding protein, SRB-RGS, binding to ERα as the first full-length RGS3 and characterized this protein in previous papers[2, 3] as well as the present study. A partial RGS3 had been characterized by

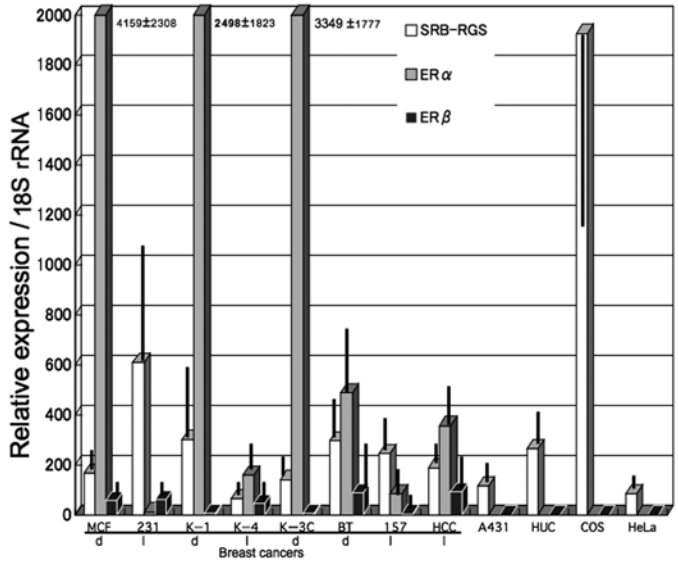


Fig. 7. Quantification of SRB-RGS, ERα and ERβ expressions in the cells by real time RT-PCR. Quantification of SRB-RGS, ERα, ERβ and 18S rRNA expressions in the cells was performed by real-time RT-PCR. The values of the SRB-RGS, ERα and ERβ gene expression were normalized by the respective value for the 18S rRNA expression. Values are the means of three or more independent experiments±SD. The bares of ERα of MCF-7 cells, MDA-MB-231 cell and KPL-3C cell were shorten to relative expression value 2000 as a matter of convenience and the real values were written beside each bar. MCF: MCF-7 cells; 231: MDA-MB-231 cells; K-1: KPL-1 cells; K-4: KPL-4 cells; K-3C: KPL-3C cells; BT: BT474 cells; 157: MDA-MB-157 cells; HCC: HCC-1937 cells; A431: A431 cells; HUC: HUC-F2 cells; COS: COS-7 cells; HeLa: HeLa cells. d: hormone-dependent cells; i: hormone-independent cells.

others[11, 14]. In our previous work, we showed that SRB-RGS was bound to the ER on DNA[3] and may suppress the ER-mediated transcriptional abilities thereby. In the present paper, the effects of SRB-RGS on the ERs-mediated transcriptional activities may be exerted by the PDZ domain-containing region and/or the RGS domain-containing region on SRB-RGS (Fig.2) as represented in Fig.8. That is, SRB-RGS may suppress the ER-mediated transcriptional activity *via* binding to ER on the DNA by the PDZ domain on SRB-RGS and/or *via* inhibition of phosphorylation of transcriptional factors containing ERs by the RGS domain on SRB-RGS. The nuclear localization signal (NLS) was in the RGS domain containing at least one region of basic amino acids (SRB-RGS aa801-967) (Figs.3, 4). The physiological conditions of the cells, such as the cell cycle and the presence or absence of hormones, may reflect the subcellular localization of SRB-RGS. Either the PDZ or the RGS domains on SRB-RGS induced cell death (Fig.5), suggesting that SRB-RGS was included in the essential survival signaling pathways. The overexpressed PDZ domain-containing protein may competitively inhibit the essential factors for survival of HeLa cells. The overexpressed RGS domain may inhibit survival signal(s) *via* inhibition of G-protein. Consequently, either the PDZ or RGS domains on SRB-RGS induced cell death. The expression was observed in both the cytoplasm and nucleus or in the cytoplasm of various types of cells by confocal laser-scanning microscopy (Fig.6). In HeLa, PC-12D, A431, rat-1, HCC1937 and MCF-7 cells, the nuclear localization ability of SRB-RGS was reduced. In COS-7, KPL-3C, MDA-MB-231, MDA-MB-157, KPL-1 and KPL-4 cells, SRB-RGS had more nuclear localization ability. Most of the former cell lines do not contain ERs. The nuclear localization ability for SRB-RGS in MCF-7 cells may be inhibited to some extent for some reason and thereby the growth of

the cells may be enhanced. The latter are mainly the breast cancer cells. In COS-7 cells, SRB-RGS may be expressed at a sufficiently high level to move into the nucleus (Fig.7).

SRB-RGS was expressed in all types of cell lines examined by the quantitative real-time RT-PCR(Fig.7). MCF-7, KPL-1 and KPL-3C breast cancer cells (hormone-dependent cells) expressed high levels of ERs, which may not be suppressed sufficiently the transcriptional activities by SRB-RGS. MDA-MB-157, MDA-MB-231, KPL-4 and HCC1937 (hormone-independent cells) and BT-474 (hormone dependent cells)[15] breast cancer cells expressed lower levels of ERs, which could be suppressed sufficiently by stoichiometrically similar level of SRB-RGS. SRB-RGS may affect the growth of this type of cells. Mutations or other functional defects of the SRB-RGS protein could enhance the cell growth. A431, HUC-F2, COS-7 and HeLa cells did not express ERs. SRB-RGS could have something to do with the growth of the cells which expressed lower level of ERs. The results by quantitative real-time PCR (Fig.7) are consistent with the observations by confocal laser-scanning microscopy (Fig.6). Taken together, either PDZ or RGS domains on SRB-RGS were necessary for transcriptional suppression, subcellular localization and induction of cell death in HeLa cells.

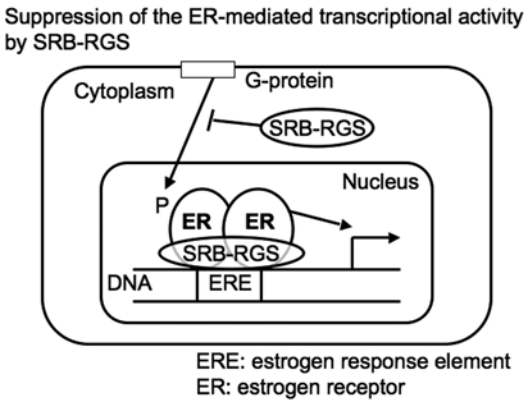


Fig. 8. Schematic representation for suppression pathways of the ER-mediated transcriptional activity by SRB-RGS. SRB-RGS may suppress the ER-mediated transcriptional activity *via* binding to ER on the DNA and/or *via* inhibition of phosphorylation of transcription factors.

Previous studies have suggested that the transcriptional factors containing ERs, microRNAs and estrogens play important roles in the breast cancer development, the hormone dependency and the tamoxifen resistance in breast cancer[16-19]. SRB-RGS may also be concerned with comprehensive cellular events through PDZ and/or RGS domains in breast cancers and other cells. The results provided the clues that helped to clarify the mechanisms of the effects of SRB-RGS to the transcriptional activity, subcellular localization, cell death and the cell growth.

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