Identification of a novel anti-apoptotic protein and characterization of mammalian regulators of G protein signaling (RGSs) in yeast

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Abstract

Regulators of G protein signaling (RGSs) are negative regulators of G protein coupled receptors (GPCRs). Our lab has demonstrated that yeast *Saccharomyces cerevisiae* is a useful system to study RGS and G protein signaling. Mammalian RGSs can be expressed in yeast and favored to interact with mammalian GPCRs as well.

Based on the observation that human RGS1 causes yeast cell growth arrest, I therefore used RGS1 expressing yeast cells to screen a mouse T cell cDNA library in order to find potential interacting proteins. From the screen, I identified a mouse sphingomyelin synthase 1 (SMS1) cDNA. By using a series of different apoptotic stimuli, such as hydrogen peroxide, osmotic stress, exogenous ceramide and its precursors, high temperature etc, SMS1 expression was found to suppress cell growth arrest and prevent viability decline, indicating that SMS1 represents an anti-apoptotic protein that functions by decreasing the intracellular level of pro-apoptotic ceramide.

Gene analysis further indicated that the SMS1 gene consists of 16 exons spread over a 256kb portion of mouse chromosome 19. It is alternatively spliced to produce 4 different transcripts (SMS1α1, SMS1α2, SMS1β and SMS1γ) and encode 3 different proteins (SMS1α, SMS1β and SMS1γ). Notably, I found that SMS1β protein does not interfere with SMS1α anti-apoptotic function, although both of these two proteins contain the protein-protein interaction domain, sterile alpha motif (SAM), at their N-terminus.

I also carried out a study to examine GPCR-RGS interactions using the yeast expression system. Our lab had noticed that there was an extra RGS5 related protein that was detected by western blot analysis in the protein extracts prepared from yeast and HEK293 cells expressing RGS5. The size of the band was approximately 2 times the molecular weight of RGS5, indicating the possibility that RGS5 forms a dimer. To further examine this hypothesis, I, therefore, performed a series of experiments, included yeast 2 hybrid assays, to demonstrate that RGS5 does interact with itself. This is the first report that RGS can form a dimer. The implications for this finding are discussed in detail.

Résumé

Les régulateurs de signalisation des protéines G (RGSs) sont des régulateurs négatifs des récepteurs couplés aux protéines G (GPCRs). Nous avons démontré dans notre laboratoire que la levure *Saccharomyces cerevisiae* est un système utile pour l'étude des RGS et de la signalisation des protéines G. Les RGS de mammifère peuvent être exprimé chez la levure et leur interaction avec des GPCR de mammifère peut également être facilitée.

Basé sur l'observation que le RGS1 humain amène un arrêt de croissance chez la levure, j'ai donc utilisé des cellules de levure exprimant RGS1 pour faire le criblage d'une librairie d'ADNc provenant de cellules T de souris afin de trouver de potentiel protéines interagissant avec RGS1. A partir du criblage, j'ai identifié un ADN de souris codant la sphingomyelin synthase 1 (SMS1). En utilisant une série de stimuli apoptotique différents comme ; le peroxyde d'hydrogène, le choc osmotique, le céramide exogène et les précurseur de céramide, la haute température etc., j'ai observé que l'expression de SMS1 empêche l'arrêt de croissance cellulaire et prévient le déclin de viabilité, ce qui indique que SMS1 est une protéine antiapoptotique qui fonctionne en réduisant les niveaux intra cellulaire de céramide qui est proapoptotique.

L'analyse du gène de SMS1 indique qu'il consiste en 16 exons répartient sur une portion de 256Kb du chromosome 19 de la souris. Il est épissé alternativement produisant 4 différents transcrit (SMS1α1, SMS1α2, SMS1β et SMS1γ) et code 3 différentes protéines (SMS1α, SMS1β et SMS1γ). Notamment,

j'ai trouvé que la protéine SMS1β n'interfère pas avec la fonction antiapoptotique d'SMS1α, même si ces protéines possèdent toute deux un domaine d'interaction protéine-protéine SAM (sterile alpha motif) en N-terminal.

J'ai aussi mené une étude afin d'examiner les interactions GPCR-RGS en utilisant la levure comme système d'expression. Nous avons noté dans notre laboratoire qu'il y avait une protéine supplémentaire apparenté à RGS5 détectée par western blot dans les extraits préparés à partir de la levure et des cellules HEK293 exprimant RGS5. Le poids moléculaire correspondant à la bande était approximativement 2 fois le poids moléculaire de RGS5, indiquant la possibilité que RGS5 forme un dimère. Afin d'approfondir cette hypothèse, j'ai donc utilisé un assai 2 hybrides afin de démontrer que RGS5 interagit avec lui-même. Ceci est la première fois que l'on rapporte la possibilité pour RGS5 de former un dimère. Les implications apportées par cette découverte sont discutées en détail.

Preface

Contribution of authors

The candidate performed the majority of the research presented in this thesis. Dr. Greenwood, as the candidate's research and thesis supervisor in the past 5 years, provided scientific direction to all studies that the candidate produced. Dr. Greenwood also assisted in the conception and interpretation of the experiments and in the writing of 3 original research manuscripts. Hence, Dr. Greenwood is co-author on all research manuscript included in this dissertation.

The contributions of other authors to this work are described as follows:

Chapter 2 is correlated with the manuscript: **Zhao Yang, Chamel Khoury, Gaël Jean-Baptiste and Michael T. Greenwood.** Identification of mouse sphingomyelin synthase 1 as a suppressor of Bax-mediated cell death in yeast. *FEMS Yeast Res.* 2006, 6(5):751-762.

Dr. Greenwood helped to construct p425GAL1-AUR1-C-GFP.

Chapter 3 is correlated with the manuscript: **Zhao Yang, Gaël Jean-Baptiste, Chamel Khoury and Michael T. Greenwood.** The mouse sphingomyelin synthase 1 (SMS1) gene is alternatively spliced to yield multiple transcripts and proteins. *Gene.* 2005, 363:123-132.

Dr. Greenwood helped to figure out the introns/exons shown in Figure 3.1 and Table 3.1; Gaël Jean-Baptiste performed western blots shown in Figures 3.5B and 3.6B. Chamel Khoury cloned SMS1β used in Figure 6.

Chapter 4 is correlated with the manuscript: **Zhao Yang, Sabrina Gaudio, Wei Song, Matthew Greenwood, Gaël Jean-Baptiste and Michael T. Greenwood.** Evidence for the dimerization of human Regulator of G-protein Signalling 5 (RGS5). *Cell. Physiol. Biochem.* 2007, 20(5):303-310.

Sabrina Gaudio designed primers of some GFP fused to different GPCR C-tails and performed western blot shown in Figure 2C; Wei Song helped with the western blots shown in Figures 4.1 and 4.4.

Other publications/contributions of the candidate

In addition to the papers included in this thesis, the candidate also contributed to the following original research studies:

Khoury C, **Yang Z**, Ismail S, Greenwood MT. Characterization of a novel alternatively spliced human transcript encoding an N-terminally truncated Vps24 protein that suppresses the effects of Bax in an ESCRT independent manner in yeast. *Gene*. 2007, 391(1-2):233-41.

The candidate extracted RNA from mouse and human tissues or cell lines, and performed tissue distribution of different Vps24 transcripts by RT-PCR shown in Figure 3.

Jean-Baptiste G, **Yang Z**, Khoury C, Greenwood MT. Lysophosphatidic acid mediates pleiotropic responses in skeletal muscle cells. *Biochem Biophys Res Commun.* 2005, 335(4):1155-62.

The candidate extracted RNA from different tissues and cell lines, and examined tissue distributions of different LPA receptor gene expressions by RT-PCR shown in Figure 1A.

Li X, **Yang Z**, Greenwood MT. Galpha protein dependent and independent effects of human RGS1 expression in yeast. *Cell Signal.* 2004, 16(1):43-9.

The candidate performed the confirmation experiments shown on Figures 2 and 3.

Contributions to original knowledge

The work presented in this thesis started from the study of RGS function, extended to sphingolipids involved in apoptosis, and finally went back to RGS by examining RGS5 dimerization. These studies have been published in peer-review journals. The major contribution of these work to original knowledge are summarized below:

1. The identification of mouse sphingomyelin synthase 1 cDNA sequence (GenBank Accession No. AY509044) by the functional screening of a mouse T cell cDNA library in yeast.
2. Provided direct evidence to show that the mouse sphingomyelin synthase 1 is a novel anti-apoptotic protein.
3. The sphingomyelin synthase 1 gene structural analysis.
4. Nomenclature of 4 sphingomyelin synthase 1 spliced transcripts and 3 encoded proteins.
5. First report the dimerizaiton between RGS proteins.

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Thanks to all members in the Greenwood lab, Gaël, Chamel, Gisselle, Sabrina and Xiaoyu, for your freely-given assistance on techniques, scientific discussion, advice as well as friendship. I really enjoyed the time when I worked with you together. I would also like to thank my advisory committee, Dr. Chantal Autexier, Dr. Andrea LeBlanc and Dr. Martin Latterich as well as PIs in the Polypeptide Hormone Lab (PPL), Dr. Barry Posner, Dr. Simon Wing and Dr. Louise Larose. I thank them for their kind guidance on my projects, brilliant advice, scientific training and support. I also want to acknowledge Mr. Gerry Baquiran for his help and support, especially with all the equipment. And also thanks to all members of PPL for their kind and generosities. Special thanks to Eric Cardin for the French translation of the abstract included in this thesis.

The most thank you will be delivered to my dear family. They gave me the strongest support and courage when I was embarrassed, panicky and full of

hesitation. Without them, I would not have been able to go through this entire 5 years of graduate school.

Finally, I thank McGill University Health Centre (MUHC) and Faculty of Medicine for funding my research.

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List of Abbreviations:

$\Delta\Psi_m$	Mitochondrial membrane potential
Aβ	Amyloid-β peptide
AbA	Aureobasidin A
AD	Alzheimer's disease
AIF	Apoptosis inducing factor
ALS	Amyotrophic lateral sclerosis
Apaf-1	Apoptotic protease activating factor 1
APP	β-amyloid precursor protein
aSMase	Acidic sphingomyelinase
BH	Bcl-2 homology
BI	Bax inhibitor
BIR	Baculovirus IAP repeat
BRET	Bioluminescence resonance energy transfer
CARD	The caspase recruitment domain
cDNA	DNA complimentary to mRNA
Cer-P	Ceramide-1-phosphate
cFLIP	Cellular-Flice like inhibitory proteins
CHX	Cycloheximide
Cyt c	Cytochrome c
DAG	Diacylglycerol
DD	Death domain
DED	The death effector domain
DIABLO	Direct inhibitor of apoptosis-binding protein with low pI
DISC	Death-inducing signaling complex
DMEM	Dulbeco's modified Eagle's media
Endo G	Endonuclease G
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated death domain protein
FRET	Fluorescence resonance energy transfer
FSH	Follicle-stimulating hormone
GABA	γ-aminobutyric acid
GAL	Galactose

GalCer	galactosylceramide
GAP	GTPase accelerating protein
GCS	Glucosyleramide synthase
GEF	Guanine nucleotide exchange factor
GFP	Green Fluorescent Protein
GleCer	Glucosyleramide
GLU	Glucose
GPCR	G-protein Coupled Receptor
GRK	G protein receptor kinase
GSH	Glutathione
HD	Huntington's disease
H_2O_2	Hydrogen peroxide
4-HPR	N-(4-hydroxyphenyl)retinamide
HRP	Horse raddish peroxidase
HSP	Heat shock protein
HtrA2	High temperature requirement protein A2
IAP	Inhibitors of apoptosis protiens
Ig	Immunoglobulin
IPC	Inositol phosphorylceramide
JNK	c-jun N-terminal kinase
kDa	Kilodalton
LH	Luteinizing hormone
LPA	Lysophosphatidic acid
MLS	Mitochondrial localization sequence
MMP	Mitochondrial membrane permeabilization
MOMP	Mitochondrial outer membrane permeabilization
MPT	Mitochondrial permeability transition
NaOH	Sodium hydroxide
NCCD	The Nomenclature Committee on Cell Death
NFT	Neurofibrillary tangle
Nma	Nuclear mediator of apoptosis
O_2^-	Superoxide
Oligo	Oligodeoxyribonucleotide
Omi	The mammalian homolog of the prokaryotic *HtrA* proteins
PA	Phosphatidic acid
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCD	Programmed cell death

PD	Parkinson's disease
PHS	phytosphingosine
PI3K	Phosphatidylinositol 3 kinase
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PLC	Phospholipase C
PS1/2	Presenilin-1 or -2
PTPC	The permeability transition pore complex
RGS	Regulator of G-protein Signaling
RLM-RACE	RNA ligase mediated-rapid amplification of cDNA ends
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
S1P	Sphingosine-1-phosphate
SAM	Sterile Alpha Motif
SDS-PAGE	Sodium dodecyl sulfate-polyacrilamide gel electrophoresis
SMAC	Second mitochondria-derived activator of caspases
SMS	Sphingomyelin synthase
SOD	Superoxide dimutase
SPH	sphingosine
SPT	Serine-palmitoyl trnasferase
TMD	Transmembrane domain
TNF-α	Tumor necrosis factor-α
TOR	Target of rapamycin
Trx	Thioredoxin
TSH	Thyroid stimulating hormone
TUNEL	Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling
UTR	Untranslated region
UV	Ultraviolet
WT	Wild type
XIAP	X chromosome-linked inhibitor of apoptosis
YCA1	Yeast caspase-like protein 1
YNB	Yeast Nitrogen Base

Chapter 1 Literature Review

1. Apoptosis and anti-apoptosis

1.1 Programmed cell death and apoptosis

1.1.1 Programmed cell death (PCD)

Programmed cell death (PCD), as a concept, was first introduced by Lockshin et al. in 1964 to describe an apparently endogenous pathway or set of pathways used by cells to commit suicide during insect development [1]. Nowadays, PCD is a well described process that involves a series of stereotypical biochemical and morphological steps leading to cell demise. These conserved pathways participate in controlling the balance between proliferation and differentiation, for example, PCD is essential for the successful completion of organogenesis and the crafting of complex multicellular tissues during embryonic development [2].

The term programmed implies that the cell death has an intrinsic character that is regulated by various intrinsic signaling pathways [3]. This type of cell death is distinct from necrosis, which only takes place when cell is subjected to some forms of trauma

[4,5].

There is no definite classification of all forms of PCD. Clarke et al. [6] describes type I (nuclear or apoptotic), type II (autophagic) and type III (cytoplasmic) cell death based on cell morphology. Others also accept the division of PCD into classical apoptosis, apoptosis-like PCD and necrosis-like PCD [3], based on nuclear morphology.

Autophagy leads to cell death when, for example, cell starves over a prolonged time. This is a type of cell survival and protective mechanism [7] by which starving cells deprived of nutrients and energy feed on degraded cytoplasmic components [8]. Autophagosomes then encircle those damaged organelles or proteins, and fuse to the lysosomes to complete the degradation of cellular components [9]. The molecular mechanism of the whole process is thought to be controlled by about 30 autophagy-related (Atg) proteins [10]. The protein kinase target of rapamycin (TOR) negatively regulates autophagy in response to both intracellular (e.g. nutrients) and extracellular (e.g. hormonal) signals [7].

1.1.2 The definition of apoptosis

Apoptosis was originally described by Kerr et al. in 1972 [11] as a morphologically uniform cell death. According to the definition of The Nomenclature Committee on Cell Death (NCCD) [12], apoptosis is "a type of cell death that is accompanied by rounding-up of the cell, retraction of pseudopodes, reduction of cellular volume (pyknosis), condensation of the chromatin, fragmentation of the nucleus (karyorhexis), little or no ultrastructural modification of cytoplasmic organelles, plasma membrane blebbing, and

maintenance of an intact plasma membrane until late stages of the process". Apoptosis is the regulated form of PCD utilized by metazoans to remove unneeded, damaged, or potentially deleterious cells [13]. It plays important roles in embryogenesis, metamorphosis, cellular homeostasis, and tissue atrophy [14]. Apoptosis operates in adult organisms to maintain normal cellular homeostasis. A good deal of research also demonstrates that the development of aggressive tumours depends on numerous defects in apoptotic signaling. This requirement for apoptosis resistance is partially explained by the nature of cancer-associated growth-promoting signals themselves [2,15].

1.2 Mechanism of apoptosis

There are three major signaling pathways of apoptosis: the extrinsic (death receptor) pathway, the intrinsic (mitochondrial) pathway, and a recently recognized endoplasmic reticulum (ER) stress pathway [9,16-18].

1.2.1 The extrinsic pathway

The extrinsic (death receptor) pathway (Figure 1.1) is initiated by the extracellular ligand binding to the cell surface death receptor. For example, trimeric Fas ligand, FasL binds to Fas, resulting in the recruitment of FADD (Fas-associated death domain protein) and inactivated caspase-8 (or caspase-10) through the receptor death domain, thus forming a DISC (death-inducing signaling complex). This formation triggers the activation of the initiator caspase, caspase-8 (or caspase-10), which then cleaves and

activates downstream effector caspases, like caspase-3, 6 and 7 [9,13,19].

Caspases, a family of cysteinyl aspartate–specific proteases, are highly conserved through evolution, and can be found from humans all the way down to insects, nematodes, hydra and yeast [20]. Over a dozen different mammalian caspases have been identified, and two-thirds of them have been suggested to function in apoptosis [14].

Caspases are synthesized as single-chain zymogens (procaspases) with a variable length prodomain followed by two conserved subunits, p20 and p10. (Figure 1.2), which are the dimer forming sites between 2 caspases [19,21]. These procaspases can form activated mature caspases by being cleaved at specific aspartate residues (proteolytic processing). Four protruding loops (L1–L4) constitute the catalytic groove of each caspase (Figure 1.2). Caspase activation is thought to be related to conformational changes in these 4 loops [19]. On the other hand, as specific cysteine proteases, caspases recognize a 4-amino acid sequence in their substrates, named S4-S3-S2-S1, which consists of the so-called tetrapeptide recognition motif. In mammals, S3 is always a glutamine. The cleavage normally takes place after S1, which is usually an asparagine. Therefore, the specificity of caspase cleavage can be described as X-Glu-X-Asp [19,21].

As mentioned above, there are 2 types of caspases, upstream caspases, called initiator caspases, like caspases-8, -9, and -10, and their downstream targets known as effector or executioner caspases, like caspases-3, -6, and -7 [13] (Figure 1.2).

The initiator caspases are found in the cytosol as monomers until dimerization is effected by adaptor molecules, such as heptameric Apaf-1 or trimeric FADD [9]. These caspases contain large structural motifs that belong to the so-called death domain

superfamily, which consists of the death domain (DD), the death effector domain (DED), and the caspase recruitment domain (CARD) (Figure 1.2). DEDs and CARDs are responsible for the recruitment of initiator caspases into death- or inflammation-inducing signaling complexes, resulting in proteolytic autoactivation of effector caspases [21].

Effector caspases have a 20–30 amino acid prodomain that is removed by cleavage, which produces a heterotetramer with 2 large subunits of 17 to 20 kDa and 2 small subunits of 10 to 12 kDa [9].

Caspases are the central executioners of the apoptotic pathway. Eliminating caspase activity, either through mutation or pharmacological inhibition, will slow down or even prevent apoptosis, but do not prevent ultimate cell death [14]. For example, inhibition of caspase activity has been shown to increase survival in liver ischaemia, decreasing 50% infarct volumes in both cardiac and focal cerebral ischaemia, and also markedly improve organ function such as renal function and neurodeficits in models of kidney diseases and focal cerebral ischaemia, respectively [22]. Caspase inhibition has also shown therapeutic promise for Parkinson's disease in preclinical animal models by decreasing apoptotic cell death [22]. Other therapeutic applications have been described, for example, by using HIV protease recognition motifs to replace the maturation sites within the caspase-3 polypeptide, HIV infected cell may undergo apoptosis when the protease clips the engineered caspase, thus selectively deleting infected cells [23].

1.2.2 The intrinsic pathway

In the intrinsic (mitochondrial) pathway (Figure 1.1), the signaling starts from the

apoptotic stimuli and the subsequent responses of mitochondria. Mitochondrial membrane permeabilization (MMP) changes induced by apoptotic stimuli, causes a number of molecules, such as cytochrome *c* (Cyt *c*), SMAC (second mitochondria-derived activator of caspases), DIABLO (direct inhibitor of apoptosis-binding protein with low pI), AIF (apoptosis inducing factor), Endo G (endonuclease G) and OMI/HTRA2 (high temperature requirement protein A2) to be released from the mitochondria intermembrane space into the cytoplasm. Among those molecules, the most studied one is Cyt *c*. Cyt *c* combines with dATP, Apaf-1 and caspase-9 to form the apoptosome in the cytosol. This complex then evokes the activation of the caspase cascade including the effector caspases, like caspase-3 and caspase-7 [13,18,19].

The apoptotic extrinsic and intrinsic pathways are not two mutually exclusive pathways. Crosstalk has been observed (Figure 1.1): the activation of the extrinsic pathway has been shown to promote the downstream actions of the intrinsic pathway. In addition to activating caspase-3, the activated caspase-8 also catalyzes the cleavage of the pro-apoptotic protein Bid yielding tBid. tBid then translocates to the outer membrane of the mitochondria and effects MMP and releases Cyt *c* and triggers caspase activation [3,13,19].

MMP is controlled by a variety of Bcl-2 family member proteins that are located or translocated to the mitochondrial outer membrane [24]. The Bcl-2 family members (Figure 1.3) are divided into three groups: anti-apoptotic proteins such as Bcl-2, Bcl-x_L, Mcl-1, Bcl-w, and A1, which contain all 4 Bcl-2 homology (BH) domains and pro-apoptotic proteins like Bax, Bak, and Bok, which consist of BH1-3, and BH3 only

proteins such as Bid, Bim, Puma, Noxa, Bik, Bmf, Bad, Hrk, and BNIP3 [18,24,25].

The pro-apoptotic members of the Bcl-2 family, like Bax and Bid, initiate MMP by forming a channel. The mechanism by which Bax and Bak induce outer membrane permeability is still controversial. In most cases, the initial apoptotic stimulus does not activate Bax directly, but rather increases the expression or activity of one or more BH3-only pro-apoptotic Bcl-2 family members like Bid and Bim. These proteins then trigger a conformation change in Bax causing it to migrate and insert into mitochondrial membranes. Once inserted into membranes, Bax then oligomerizes to permeabilize the membranes. Bak can also loosely associate with mitochondrial outer membrane. Bax and Bak then form pores that allow the release of proteins across the outer mitochondrial membrane [26-28].

Recently, Dlugosz et al. [29] proposed a model to explain the mechanism by which Bcl-2 suppresses the ability of Bax to cause the release of proteins from the mitochondria. According to the model, anti-apoptotic Bcl-2 is normally bound to mitochondrial membranes, while pro-apoptotic Bax is an inactive monomer located in the cytoplasm or loosely bound to mitochondria. Upon the apoptotic stimuli, death signals activate BH3-only proteins (tBid, Bim), which cause changes in the conformations of both Bax and Bcl-2. Only those conformationally changed Bcl-2 proteins can inhibit the oligomerization of Bax and prevent it from permeabilizing membranes and releasing pro-apoptotic factors.

1.2.3 Some other important PCD triggers

In addition to the Bcl-2 protein family, other agents, such as ROS (reactive oxygen species), ceramide and Ca^{2+} also serve to promote the mitochondrial permeability transition (MPT) [13].

ROS is a term that consists of a wide range of molecules, including oxygen free radicals and any other oxygen-containing molecule in which an oxygen atom has a greater reactivity than molecular O_2 [30]. Free radicals are chemical species containing one or more unpaired electrons. The unpaired electrons of oxygen react to form partially reduced highly reactive species that are classified as ROS, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical, and peroxynitrite [31].

ROS can be generated in many ways. Mitochondrial oxygen metabolism is thought to be the predominant source of superoxide radicals in cells [31,32]. Oxidative stress resulting from an imbalanced ratio between ROS production and detoxification may disturb physiological signal transduction, lead to chain reactions in lipid layers, mediate DNA damage, and also initiate apoptosis and oncogenesis [13,33]. As an example, malonate, an inhibitor of mitochondrial complex II, has been shown to produce ROS in human SH-SY5Y neuroblastoma cells, leading to oxidative stress, Cyt *c* release, and apoptotic cell death [34]. This is because ROS subsequently mediates p38 MAP kinase activation and causes the activated Bax to induce MMP leading to neuronal apoptosis [35].

Antioxidant enzymes, such as phospholipid hydroperoxide glutathione peroxidase, glutathione peroxidase and manganese superoxide dismutase, regulate ROS accumulation in mitochondria. By reducing glutathione (GSH), mitochondria may against peroxides

generated from the electron transport chain through the GSH redox cycle [32].

Another important apoptotic related agent is ceramide, the most studied pro-apoptotic sphinoglipid, which is a well known stress response mediator [36,37]. Ceramide can be accumulated by (i) *de novo* synthesis from L-serine and palmitoyl–CoA mediated by serine-palmitoyl transferase (SPT) [38]; (ii) conversion from sphingomyelin hydrolysis [39]; (iii) inhibition of ceramide hydrolysis; and/or (iv) stimulation of glucosylceramide hydrolysis or inhibition of its synthesis [40]. For example, acidic sphingomyelinase (aSMase) catalyzes the generation of ceramide which mediates Bax conformation change and induces MMP *in vitro* and in HeLa cells [41]. Similar results were seen in experiments with endothelial cells. aSMase deficient mice shows high resistance to radiation induced cell death, indicating that ceramide generation by aSMase may be important in microvasculature homeostasis in addition to cancer cell homeostasis [42,43].

Many apoptotic stimuli such as stress, radiation, chemotherapy, and death ligands modulate ceramide generation and cause ceramide accumulation. Subsequently, ceramide regulates many downstream intracellular effectors including phosphatases, proteases, and protein kinases that mediate apoptotic activations [37]. For instance, the tumor necrosis factor (TNF) receptor is the key mediator to TNF signaling. TNF binding induces rapid endocytosis of the activated TNF-R1 complex (TNF receptosomes) via clathrin-coated vesicles. After the recruitment of TRADD, FADD, and caspase-8, TNF receptosomes fuse with trans-Golgi vesicles which contain pro-aSMase to form multivesicular endosomes in which aSMase is activated [44].

1.2.4 ER-stress pathway

The third major apoptotic pathway in cells is mediated by ER stress. Ca^{2+} storage and signaling as well as folding, modification, and sorting of newly synthesized proteins are among the main functions of the ER. Disturbance of any of these functions can lead to ER stress [45,46]. Mild or initial ER stress responses are cytoprotective. It restores ER dysfunction disrupted homeostasis by attenuating protein synthesis, increasing folding capacity by inducing chaperone expression, and improving ER-associated degradation (ERAD) ability with the expression of ERAD components. When this dysfunction persists, ER stress can initiate apoptosis [16,47].

Like most of apoptotic pathways, the ER stress mediated apoptotic pathway is also caspase dependent. A specific caspase, caspase-12, is not activated by other cell death stimuli except for ER stress [48]. This pathway subsequently activates the common caspase cascade and connects with the mitochondrial pathway [17]. Therefore, the activation of caspase-12 can be reversed by overexpression of Bcl-2 [49]. On the other hand, ER stress inducer, Brefeldin A (BFA) causes the pro-apoptotic BAD dephosphorylation and decreases Bcl-2 expression [50]. Morishima et al. demonstrated that, on the ER surface, Bim accumulation induced apoptosis which depended on caspase-12 activation [51].

ER stress can up-regulate the expression of hundreds of genes, including autophagic genes ATG8, ATG14 and APE1 [52]. By using baker's yeast *Saccharomyces cerevisiae* as an experimental system, Klionsky's group reveals that ER stress involves in the assembly of the pre-autophagosomal structure. Transportation of autophagosomes to the

vacuole is stimulated in an Atg protein-dependent manner, and Atg1p has high kinase activity during ER stress-induced autophagy similar to the situation in starvation-induced autophagy, indicating that ER stress is a new pathway to trigger autophagy [53,54]. The link between ER stress and autophagy is also being investigated in other systems [55-57].

1.3 Regulation of Apoptosis

There are the large number of proteins that can make a cell undergo apoptosis, conversely, there are also exists a large number of proteins that are anti-apoptotic and prevent cell death. Anti-apoptotic members of the Bcl-2 family are probably the classic examples. Overexpression Bcl-2 and Bcl-x_L can attenuate ceramide accumulation and prevent subsequent Cyt *c* release [58,59].

Inhibitors of apoptosis proteins (IAP) are another well studied group of anti-apoptotic proteins [60,61]. In addition to containing baculovirus IAP repeat (BIR) domains, members in this group also consist of other structural domains, e.g. a RING or caspase activation recruitment domain (CARD) [62]. The best characterized member of IAP is XIAP (X chromosome-linked inhibitor of apoptosis). XIAP prevents apoptosis in both caspase-dependent and -independent manners. XIAP inhibits caspase activity by interacting with caspase-9 through its BIR3 domain, whereas its BIR2 domain inhibits caspases-3 and -7 [19,63]. It can also promote proteosomal degradation by marking proteins with ubiquitin molecules, because the RING domain of XIAP has E3 ubiquitin

ligase activity [64].

Some pro- and anti-apoptotic proteins share common domains that can serve as signatures for identification of proteins involved in the network [65]. For example, the death effector domain (DED) is present in some caspases. The anti-apoptotic function of some DED containing proteins such as cellular-Flice like inhibitory proteins (cFLIPs) is likely mediated by their ability to interact with other DED containing caspases [66].

Other apoptotic inhibitory proteins include Heat Shock Proteins (HSPs), which respond to stress and rescue cells from the apoptotic signaling cascades. HSP works as chaperones and their anti-apoptotic effects are likely due to their ability to maintain protein structures during stress [67]. As an example, Hsp70 has been shown to be critically involved in the protection against cellular injury in pancreatic acinar cells by the Saluja group [68,69]. Recently, the same group reported that increased levels of Hsp70 also lead to apoptotic resistance of pancreatic cancer cells [70]. A variety of antioxidant proteins are also anti-apoptotic, such as superoxide dismutase (SOD) and thioredoxin (Trx), as they decrease the levels of stress induced ROS. Ceramide ultilizing enzymes such as SMS1 also prevent apoptosis by decreasing the levels of the pro-apoptotic sphingolipid [37,39,71].

1.4 Apoptosis and diseases

1.4.1 Apoptosis and cancer

The accumulation of too many cells in cancer is the result of excessive cell proliferation and/or insufficient apoptosis. However, there are some evidence [72,73] that indicates that apoptosis may not be the only mechanism by which cancer cells undergoe cell death when undergoing genotoxic stress. Autophagic degradation may also be related to some cancers. For example, constitutive activation of phosphotidylinositol-3-kinase (PI3K) signaling is common in human cancer cells and partially controls the autophagic pathway [74]. PI3K and its downstream effectors, AKT and TOR, normally suppress autophagy, where as a tumor suppressor, PTEN, which is a negative regulator of PI3k signaling, promotes autophagy [75,76].

On the other hand, the inactivation of apoptosis also plays important roles in tumorigenesis. This disabling of apoptotic responses might be a major contributor to chemo- and radio-therapy resistance [77,78]. Glucosylceramide synthase (GCS) converts pro-apoptotic ceramide to generate nonfunctional glucosylceramide. This happens in many tumor types and causes multidrug resistance [79]. Therefore, chemotherapy efficiency will be improved by restoring apoptotic signaling in tumor cells.

p53 is an example of a well known tumor suppressor gene [30]. Upon apoptotic stimuli, including DNA damage or hypoxia, p53 rapidly translocates to the mitochondria where it initiates MOMP (mitochondrial outer membrane permeabilization) by neutralizing anti-apoptotic proteins (e.g., Bcl-x_L) and activating the pro-apoptotic Bax or Bak [80,81]. This leads cells to either cell-cycle arrest or to apoptosis [82]. In addition, p53 mutations or defects are involved in most human cancers, while cells expressing mutant p53 are sensitive to drug-induced apoptosis [83]. The higher sensitivity of developing cancers in p53-null mice also supports the view that inactivation of the

apoptosis pathway is necessary to allow cancer cells to develop [84]. On the other hand, the presence of wild type p53 does not necessarily indicate that the p53 pathway is intact. It is possible that defects of p53 or altered expression of upstream p53 regulators can also occur [24].

1.4.2 Apoptosis and cardiovascular disease

Apoptosis has been detected in the myocardium in a number of cardiac pathologies including hypoxia, ischemia followed by reperfusion, myocardial infarction, myocardial hypertrophy, and end-stage heart failure. Apoptosis has also been detected in atherosclerotic lesions of the vasculature [85].

Heart failure, a major cardiovascular health problem, is characterized by adverse structural changes (cardiac remodeling) and an inexorable progression of the disease that continues to occur even after the initial injury has abated. Disease development is first associated with hypertrophy, followed by an obvious reduction of cardiomyocyte numbers. This loss of cardiomyocytes in failing or cardiomyopathic hearts has been shown to involve apoptotic cell death [86].

The interesting thing is that although a large number of cardiomyocytes display Cyt *c* release and caspase-3 activation, cells are not reported to have nuclei fragmentation and the typical apoptotic morphology [87]. This phenomenon is called *apoptosis interruptus* [88,89]. The possible explanation of this is that the failing cardiomyocytes seem to invoke a number of protective mechanisms geared to limit damage when the apoptotic process is activated. For example, in one study of end-stage human heart failure [88],

XIAP (an inhibitor of caspase-9 and caspase-3) and Smac-S (a competitor of the anti-apoptotic Smac-L) are upregulated, while expression of the anti-apoptotic Smac-L (an inhibitor of XIAP) is down. The increased XIAP expression results in the inhibition of caspase-9 activity, which interferes with the enzyme's downstream effects on protein fragmentation [90,91]. In addition, the expressions of Bcl-2 and Bcl-x are significantly upregulated, which could limit Cyt *c* release from the mitochondria [91].

1.4.3 Apoptosis and neurodegenerative disorders

In addition to playing an important role in early neurogenesis, apoptosis is currently regarded as the main form of death during neurodegenerative disorders [92]. Excessive death of one or more populations of neurons results in disease or injury. For example, death of hippocampal and cortical neurons is associated with Alzheimer's disease (AD), death of midbrain neurons results in Parkinson's disease (PD), death of neurons in the striatom is associated with Huntington's disease (HD) and finally, death of lower motor neurons is associated with amyotrophic lateral sclerosis (ALS) [78].

AD is "a progressive neurodegeneration characterized by a disruption of synaptic function leading to a remarkable cognitive decline, including memory impairment and behavioural changes. The pathological hallmarks of AD include the presence of aggregates of β-amyloid peptide in neuritic plaques and intracellular neurofibrillary tangles (NFTs) of hyperphosphorylated tau (PHFtau), together with loss of neurons and synapses in the neocortex, hippocampus and other subcortical regions of the brain." [92,93]. These features are present both in sporadic AD and in early-onset familial form

(FAD) which has been associated with genetic mutations of the β-amyloid precursor protein (APP), presenilin-1 and -2 (PS1 and PS2) genes [94]. Increased DNA damage and caspase activity as well as altered expression of Bcl-2 family members have been demonstrated in neurons associated with amyloid deposits [95]. Moreover, caspase-mediated cleavage of APP results in the release a carboxy-terminal peptide, which is a potent inducer of apoptosis [96,97].

PD patients suffer from degeneration of dopamine-dependent neurons in their substantia nigra. Increase oxidative stress and mitochondria dysfunction seem to be the central key of this disease [98]. Strong caspase-3 activity as well as increased expression of other apoptosis-related genes, including p53, CD95, Bax and Par-4, has also been observed in brain tissue from PD patients [99-101].

1.5 Yeast as a model system to study apoptosis

1.5.1 Yeast apoptosis

Saccharomyces cerevisiae, the best-studied yeast, was the first eukaryotic organism whose complete genome was sequenced in 1996 [102,103]. The failure to identify yeast homologues of Bcl-2 family proteins in the yeast genome [104] raised doubts that yeast undergo programmed cell death [105]. This controversy nevertheless did not prevent yeast from being used as an effective tool to study apoptosis. The expression of mammalian pro-apoptotic Bax in yeast was found to cause cell death, which was

confirmed to be apoptotic like by TUNEL assay, Annexin V staining and detection of chromatin condensation [106]. Yeast has been used to identify many pro- and anti-apoptotic proteins. The above mentioned Bax expression system has been used as a screening system to identify a large number of anti-apoptotic proteins, such as interaction-relevant domains of Bcl-2 [107], human BI-1(Bax inhibitor-1) [108], prion [109], Ku70 [110], Vps24 [111] etc.

As early as 1997, Madeo et al. reported a *S. cerevisiae* mutant in the cell division cycle gene *CDC48* (*cdc48*^{S565G}) showed an apoptotic phenotype with typical hallmarks of mammalian apoptosis, including DNA degradation, chromatin condensation as well as externalization of phosphatidyl serine to the outer plasma membrane leaflet, implicating the existence of apoptosis-like cell death in yeast [112]. More and more evidence shows that, like in mammalian cells, yeast cells undergo apoptosis in response to several environmental stimuli. These include oxidant stress, such as hydrogen peroxide [113-115], acetic acid [116]; hyperosmotic stress, such as elevated concentrations of glucose [117], sorbitol [118,119] and NaCl [120,121]; temperature changes [122-124] as well as DNA damage caused by UV [125] or different drugs, like Adozelesin, Amphotericin B and Aspirin [125-127].

1.5.2 Yeast apoptotic proteins

1.5.2.1 Yeast caspase-like protein (Yca1p)

Madeo et al. [128] reported a metacaspase member in *S. cerevisiae*, Yor197w, which

can be cleaved in a caspase-typical way and displays a caspase-like proteolytic activity. Disruption of Yor197w increases tolerance to hydrogen peroxide-induced apoptosis whereas overexpression of Yor197w triggers an increased sensitivity to hydrogen peroxide-induced apoptosis as well as an increase in the activation of the Yor197w protein. Because of these properties, Yor197w was named as yeast caspase 1 (*YCA1*).

The involvement of Yca1p in different kinds of yeast death has been extensively studied [129-134]. In most experiments, the importance of caspase-like activity was determined by using different inhibitors and/or yeast strains having the *yca1* deletion (*Δyca1*). For example, by comparing wild type (WT) and *Δyca1* strain, Guaragnella et al. demonstrated that Yca1p is at least partially necessary to induce yeast cell death by acetic acid [135]. In addition, Yca1p is also essential for hydrogen peroxide induced apoptosis by down-regulation the 20S proteasome activity which is involved in degradation of oxidized proteins [130], and Yca1p is a downstream executor of cell death induced by mRNA perturbations as well [131].

Although caspase-independent apoptosis exists in yeast [29,136], Yca1p looks to play a central role in yeast apoptosis [137]. However, there are still a large number of details about Yca1p that need to be elucidated. As an example, the target substrates that are cleaved by this putative protease have not been identified [105]. If YCA1 encodes the executioner caspase that is analogous to caspase-3, clues might come from studies of this caspase in mammalian cells. For example, cleavage of actin as well as a number of other protein substrates is responsible for the death inducing effects of caspases in mammalian cells [133].

1.5.2.2 Apoptosis inducing factor (AIF)

The apoptosis inducing factor (AIF) homologue is another conserved protein of the apoptotic machinery from yeast to human [138]. AIF is synthesized as a 67 kDa precursor, imported into mitochondria via its N-terminal prodomain containing two mitochondrial localization sequences (MLS) and then processed to be a 62 kDa mature protein [139]. Upon apoptotic stimuli, Cyt *c* release activates caspases, leading to the loss of mitochondrial membrane potential ($\Delta\Psi_m$), resulting in the swelling of the matrix, MOMP and rupture of the outer mitochondrial membrane and allowing the release of larger proteins such as AIF. Free AIF then translocates to the nucleus [3] where it triggers PCD by its integrated C-terminal domain [140,141]. AIF is thought to be mainly responsible for chromatin condensation and cleavage of DNA into high molecular weight fragments (50–300 kb) [142].

Similar to mammalian AIF, the yeast AIF homologue localizes to the mitochondria and translocates to the nucleus of yeast cells in response to H_2O_2 induced apoptosis [137]. Interestingly the apoptotic function of Aif1p in yeast seems to be in partly Yca1p dependent, as cell survival during overexpression of Aif1p together with mild H_2O_2 stress was elevated from 10 to 70% when *YCA1* was deleted [137].

1.5.2.3 Omi/Htra2 -like protein (Nma111p) and Bir1p

In mammals, Omi/HtrA2 is a mitochondrial pro-apoptotic serine protease that is able

to induce both caspase-dependent and caspase-independent cell death [143]. Htr (High Temperature Requirement) A2 is a member of HtrA serine protease family that is essential for bacterial survival from heat shock or tunicamycin treatment [144]. Omi is the mammalian homolog of the prokaryotic *HtrA* proteins [95]. Omi/HtrA2 is formed as a precursor that translocates to the mitochondria, where it is processed to its mature form by proteolytic cleavage. Stress signals stimulate Omi/HtrA2s release to the cytosol, where it induces apoptosis by its protease activity and by its physical and antagonistic interaction with XIAP [145].

Recently, Fahrenkrog et al. [146] used a yeast 2 hybrid screen to identify Nma111p (Nuclear Mediator of Apoptosis, ~111 kDa protein), an HtrA-like nuclear serine protease in *S. cerevisiae*. Cells lacking *nma111* (*Δnma111*) show increased survival at elevated temperatures or H_2O_2 treatment, whereas overexpression of Nma111p leads to enhanced apoptotic-like cell death, indicating that Nma111p acts as a mediator of yeast apoptosis. Meanwhile, results show that the point mutation, ProtA-Nma111p-S235C, and *Δnma111* containing strains show better cell survival than WT, indicating that serine235 is required for the death-promoting activity of Nma111p, and also implying that this activity depends on Nma111p serine-protease activity.

Mammalian Omi/HtrA2 functions by interaction with IAPs, which are characterized by a ~70 amino acids BIR (baculoviral IAP repeat) domain. The only IAP-like protein in *S. cerevisiae* is Bir1p, which has been shown to be involved in meiosis and in chromosome segregation rather than yeast apoptosis [146]. However, Walter's group [147] reported that cytoplasmic and nuclear protein Bir1p is essential to protect cells from H_2O_2 induced apoptosis in yeast. This protection can be antagonized *in vivo* by

simultaneous overexpression of Nma111p, indicating that Bir1p, like its mammalian homologue Survivin, is the substrate for Nma111p. Data indicating that Bir1p is unable to interact with Yca1p directly, suggests that the Bir1p anti-apoptotic function is indirect.

1.5.2.4 Endonuclease G (Endo G)

By using a reaction system that reconstitutes *in vitro* purified mitochondria, recombinant Bid and recombinant caspase-8, Wang's group [148] identified a mitochondrion-specific nuclease, endonuclease G (Endo G). Endo G has been thought to participate in mitochondrial replication and co-localize with Cyt *c* in the intermembrane space of mitochondria [148-150]. When apoptotic stimuli induced caspase-8 cleaves Bid, the truncated Bid (tBid) translocates to the mitochondria and releases Endo G together with Cyt *c*. After its release, Endo G cleaves chromatin DNA into nucleosomal fragments independently of caspases [148,151].

Interestingly, Zassenhaus et al. cloned the *S. cerevisiae* nuclear gene, NUC1, which encodes the major mitochondrial nuclease. However, the NUC1 knock out strain has no specific phenotype compared with the WT stain [152]. The results of amino acid sequence alignments indicates that NUC1 shows 42% identity and 62% similarity to human Endo G, implying yeast Nuc1p, a protein of 37 kDa encoded by the ORF YJL208c, is a sequence homolog of mammalian Endo G [153]. Madeo's group studied the regulative role of Endo G in yeast cell death and survival [153]. Overexpression of NUC1 made cells sensitive to low doses of H_2O_2 treatments, accompanied by enhanced ROS production and an increase in apoptotic DNA fragmentation. On the contrary,

depletion of NUC1 (*Δnuc1*) did not protect cells from different apoptotic stimuli, like H_2O_2, acetic acid, or amiodarone, but sensitized cells to cell death in standard culture conditions (glucose, synthetic media and logarithmic growth), even without additional apoptotic stimuli. Further investigations indicated that overexpression of NUC1 kills cells independently of AIF1 and YCA1. Instead, this death depends on physical interactions with potential components of the permeability transition pore complex (PTPC), the karyopherin KAP123 and phosphorylation of H2B.

2. G protein coupled receptors (GPCRs) and regulators of G protein signaling (RGSs)

2.1 G protein coupled receptors (GPCRs) and G protein signaling

2.1.1 Introduction to GPCRs

G protein coupled receptors (GPCRs) are one of the largest mammalian protein families with over 800 members which occupy more than 2% of the genes encoded by the human genome [154,155]. GPCRs are also present in the genomes of yeast, plants, nematodes and etc., indicating that receptors are highly conserved [156].

GPCRs are mostly located on the plasma membrane. Well known common features of GPCRs include 7-transmembrane α-helix domains (TMDs), an extracellular amino terminus and an intracellular carboxyl terminus. There are protein fragments, called loops that connect each TMD (TM I - VII) inside (i1, i2 and i3) and outside (e1, e2 and e3) the plasma membrane. These loops are important in stabilizing receptor conformation, serving as ligand binding sites and signal transduction sites, thus helping the receptor in signaling [157]. Actually, GPCRs serve key regulatory functions for a large number of biological processes as diverse as vision, smell, blood clotting, physiological responses to numerous hormones and the regulation of blood pressure [118].

After GPCRs are synthesized, they initially reside in the endoplasmic reticulum (ER),

where they are folded into proper functional conformations with the assistance of chaperones [158]. In addition, oligomerization, either homo- [159,160] or heteromeric [161,162] GPCR structures also likely form in the ER [163]. Following ER export, GPCRs transit through the Golgi apparatus. During this migration, receptors undergo post-translational modifications (e.g. glycosylation) to attain mature status. Mature GPCRs with the additional modifications, like the addition of oligosaccharides, then move toward their functional destination, the plasma membrane [163,164].

GPCR can be classified into three distinct families, A, B and C, based on the sequence similarity within 7 TMDs [157]. Family A is the largest group, it includes rhodopsin, odorant receptors, adrenaline (adrenergic receptors), some receptors for small peptide hormones, and the large glycoprotein hormones, thyroid stimulating hormone (TSH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). Family B shows no sequence homology to A. It contains only ~25 members, including the receptors for the gastrointestinal peptide hormone family, corticotropin-releasing hormone, calcitonin and parathyroid hormone. All receptors in this family seem to couple mainly to activation of the effector adenylyl cyclase through the G protein $G\alpha_s$. Family C members include the metabotropic glutamate receptors, an extracellular Ca^{2+}-sensing receptor, and putative taste and pheromone receptors [165,166].

2.1.2 GPCR activation and G protein signaling

GPCRs serve as mediators that convert signals from the extracellular environment into intracellular effects in a wide array of different cell types. In order to do this, GPCRs

initially are activated by binding to receptor specific agonists, that range from biogenic amines, like noradrenaline, dopamine and histamine; amino acids and ions, like glutamate, calcium and GABA (γ-aminobutyric acid); lipids, like LPA (lysophosphatidic acid), S1P (sphingosine-1-phosphate) and leukotrienes; peptides and proteins, like chemokines, angiotensin, thrombin, etc.; and others, like light or odorants [154,156,157].

Following the ligand binding, receptor conformation is changed and this facilitates activation of the heterotrimeric G protein that consists of α, β and γ subunits. There are active and inactive states of the heterotrimeric G proteins. In the inactive state, GDP is bound to α subunit that is tightly associated with β and γ subunits. Upon agonist stimulation, the receptor acts as a guanine nucleotide exchange factor (GEF), promotes GTP to replace GDP on the α subunit, and this completes the transfer from the inactive to the active state. The α subunit and the βγ complex are now able to activate or inhibit downstream effectors respectively, leading to receptor, G protein and cell-type specific characteristic signaling responses [167-169].

G protein α subunits can be divided into 4 subfamilies: $G\alpha_s$ stimulates adenylyl cyclase; $G\alpha_{i/o}$ inhibits adenylyl cyclase and regulates ion channels; $G\alpha_{q/11}$ activates phospholipase Cβ (PLCβ); and $G\alpha_{12/13}$ regulates Rho GEF related to p115 [170,171]. Gβγ subunits may activate PLCβ, PI3Kγ (phosphatidylinositol 3-kinase γ) as well as ion channels [154].

Gα and Gβγ subunits not only regulate those second-messenger generating systems, but also control the activity of intracellular signal-transducing molecules, such as the Ras and Rho families of proteins, extracellular signal-regulated kinase (ERK), c-jun N-

terminal kinase (JNK), p38 and etc. [154].

2.1.3 Termination of GPCR responses

The activated receptor is recognized and phosphorylated by a G protein Receptor Kinase (GRK) [172-174]. This leads to a decrease in receptor mediated activation of heterotrimeric G proteins. The phosphorylated receptor then recruits β-arrestin, leading to the internalization of the receptor, and the internalized receptor has three possible fates: it can recruit and activate other proteins, such as Src, leading to the activation of different signaling responses; it can be degraded likely via the lysosome; or it can be recycled back to the cell surface where it can bind ligand once again [175,176].

Deactivation of G protein-mediated signaling occurs via the inherent GTPase activity of the Gα subunit, which causes hydrolysis of GTP, and subsequent reassociation of the G protein heterotrimer. The RGS protein family largely regulates Gα-protein deactivation, essentially increasing the rate of GTP hydrolysis and favoring heterotrimer reassociation [118,177,178].

2.1.4 GPCRs as drug targets

In addition to the large number and widespread distribution, GPCRs are involved in almost all major diseases including cardiovascular, metabolic, neurodegenerative, psychiatric, cancer and infectious diseases [179-184]. They also play well-recognized roles in clinical medicine. For example, more than 50% of drugs target GPCRs and their

annual worldwide sales exceed 50 billion dollars [185]. Notable examples include Eli Lilly's Zyprexa (mixed serotonin/dopamine antagonist), Pfizer's Neurontin ($GABA_B$ agonist), GlaxoSmithKline's Imigran (Serotonin agonist), and Novartis's Diovan (Angiotension II (AT_1) antagonist). Each of them have sold more than 1450 million US dollar in the world [155].

Mutations in GPCRs can elicit a wide spectrum of disease phenotypes and/or differential drug efficacies. Mutations, leading to loss-of function or gain-of-function that can alter the receptor's ability to be activated, to couple to heterotrimeric G proteins, to bind ligand or to become desensitized. This results in hormone resistance and clinically significant impairment of signal transductions [186,187]. Loss-of-function mutations are inactivate mutants that prevent signaling in response to the corresponding agonist whereas gain-of-function mutations are enhanced mutants that lead to constitutive, agonist-independent activation of signaling, which serves to mimic conditions with hormone excess [166].

Diseases with GPCR mutations have been identified in patients with various disorders of the pituitary–thyroid, pituitary–gonadal and pituitary–adrenal axes, as well as abnormalities of food intake, growth, water balance and mineral-ion turnover [186]. For example, Melanocortin 4 receptor (MC4R) plays an important role in body weight regulation by being involved in hypothalamic control of food intake. Loss of MC4R function is generally caused by heterozygous missense mutations, which segregate with obesity and show incomplete penetrance. Since it is known that MC4R activation generates intracellular cAMP, knowledge of molecules that can affect cAMP generation or otherwise mimic MC4R-induced signaling could provide targets for novel anti-obesity

drugs [186,188].

Cardiovascular disease is the leading cause of death and illness. There are over 200 cardiac GPCRs [182]. In addition, GPCRs are also linked inflammation to cancer, modulate UV-induced DNA damage, and play a crucial role in cancer growth and metastasis. Agonists and antagonists of GPCRs are widely used in the treatment of diseases that effect major organ system including the CNS, respiratory, metabolic and urogenital systems [154]. All these studies highlight the clinical value of targeting GPCRs.

2.2 Regulators of G protein signaling (RGSs)

2.2.1 General introduction

As mentioned above, ligand bound GPCRs work as GEFs to activate plasma membrane associated heterotrimeric G proteins. The GTP-bound activated Gα proteins is inactivated when the GTP is hydrolyzed by the intrinsic GTPase activity of the Gα protein. This GTPase activity is also be enhanced by Regulators of G protein signaling (RGSs).

An early indication that the G protein cycle might be regulated by extrinsic factors came from the observation that the product of the yeast *SST2* gene could negatively regulate heterotrimeric G protein signaling [189,190]. By 1996, a large number of proteins in *Caenorhabditis elegans* and mammalian cells with similar structures were

identified and called Regulators of G-protein Signalling (RGSs) because of their ability to attenuate GPCR signaling [191,192]. RGSs are GTPase accelerating proteins (GAPs) that serve to hydrolyze the GTP on the active Gα subunit, switch the Gα subunit from an active to an inactive state, thus they terminate G protein mediated effector activation [118,169,193]. RGSs can also function as effector antagonists by preventing activated Gα proteins from interacting and activating their effector proteins. RGSs are a protein family that contains a conserved RGS domain (about 130 amino acids).

The sizes of the different RGS-containing proteins show significant divergence ranging from small RGS proteins that contain little more than an RGS box to the large RGS-containing proteins that have identifiable functional sequence motifs, such as PDZ, GGL and DEP domains (Figure 1.4). These non-RGS regions or domains are one of the more obvious characteristics that confer specific functions on the different RGSs. They either directly or indirectly influence how other proteins interact with RGSs [118,177]. For instance, by examining the yeast RGS protein Sst2 and its cognate GPCR (Ste2), Thorner's group [194] recently demonstrated that the DEP domain is as necessary for Sst2 function as its hallmark RGS domain. The DEP domain was also found to be necessary and sufficient to direct Sst2 selectively to the STE2 mediated pheromone response pathway. Anchoring of Sst2p to the Ste2p receptor by its DEP domain ensures that the Sst2 RGS domain is situated in close proximity to its substrate, permitting immediate and efficient action for Sst2 binding to its Gα protein targets.

On the basis of sequence identity and the presence of shared domains, RGS proteins have been classified into a number of different subgroups or families (Figure 1.4). They include the A/RZ, B/R4, C/R7, D/R12, E/RA, F/GEF, G/GRK and H/SNX subfamilies

[118,177,178] (Figure 1.4). Most RGS proteins are GAPs for $G\alpha_{i/o}$ family members and many also act on $G\alpha_{q/11}$ proteins, but none affect the GTPase rates of either $G\alpha_{12/13}$ or $G\alpha_s$ family members [195]. Nevertheless RGSs can block $G\alpha_s$ mediated signaling by serving as an effector antagonist and preventing $G\alpha_s$ mediated activation of effectors such as adenylyl cyclase [196].

2.2.2 Regulation of RGSs

RGS proteins are highly regulated by various mechanisms including alterations in expression levels, subcellular localization, post-translational modifications and binding to different proteins. Increases or decreases in cellular levels of RGS proteins have the potential to be critical for RGS-induced regulation of G protein signaling [118,177,197]. This is because endogenously expressed levels of RGSs appear to be rate limiting for their ability to inhibit GPCR responses. As an example, in the yeast *Saccharomyces cerevisiae*, Sst2p is the RGS that regulates signaling from the *STE2* encoded receptor for the α-factor pheromone. Overexpression of *SST2* decreases Ste2p receptor responses while cells lacking a *SST2* gene, show a hyperresponsive Ste2p receptor [190].

There is evidence which demonstrates that RGS protein and mRNA levels are dynamically altered by various drugs, second messengers, and disease states [197-200]. For instance, Dopamine D1 receptor agonists increase RGS2 mRNA expression, whereas the dopamine D2 receptor activation decreases it [201]. Morphine or cocaine treatment changes the expression of RGS4 mRNA in the rat brain [202]. Altered levels of RGS protein and/or mRNA have also been reported in a number of models representing

aspects of human behavior or disease including sepsis, kindling, spinal cord injury and stress [177,197] as well as Bartter's/Gitelman's syndrome, the failing heart, schizophrenia, Parkinson's disease and Alzheimer's disease [118]. Altered levels of RGSs will likely lead to alterations in GPCR signaling responses. For example, correlating with other reports, we have shown that RGS levels are increased in the heart of septic animals [203]. This increase is likely responsible, at least in part, for the decrease in GPCR responses associated with sepsis [118,204].

RGS proteins are known to be regulated by a variety of post-translational modifications such as palmitoylation, phosphorylation and sumoylation, which serve to modulate RGS localization, stability and GAP activity [118,177,197]. Among those modifications, phosphorylation is the most studied. A number of serine/threonine and tyrosine kinases are likely responsible for the phosphorylation of RGSs including protein kinase C (PKC), protein kinase A (PKA), protein kinase G (PKG), casein kinase 2, Src, ERK, etc. [177,205]. RGS proteins may be substrates for more than one kinase and these may have differential effects. For example, RGS2 is a substrate for both nitric oxide-activated cGMP-dependent protein kinase I-α (PKGI-α) and PKC [177]. PKC phosphorylated RGS2 reduces GAP activities and leads to increased $G\alpha_q$ mediated activation of inositol lipid signaling [206].

In addition, regulation of RGS proteins can also occur via binding to other proteins. For instance, Benzing et al. demonstrated that 14-3-3 proteins bind RGS7 on phosphoserine 434 within the RGS domain, thereby decreasing RGS7 GAP activity [207]. On the other reports, 14-3-3 proteins bind RGS3 at phosphoserine 264 in a region outside the RGS domain, but they still serves to reduce the potency of RGS3 in the inhibition of

G protein signaling [197,208]. Chidiac's group recently reported that two isoforms of 14-3-3 protein, β and ε, directly interact with purified RGS 4, 5 and 16 proteins. 14-3-3 inhibits the GTPase activity of RGS4 and RGS16, but has limited effects on RGS5 in vitro, while 14-3-3ε sequesters RGS4 in the cytoplasm and impedes its recruitment to the plasma membrane by Gα protein in HEK 293 cells [208,209].

Phosphatidylinositol-3,4,5,-trisphosphate (PIP_3), phosphatidic acid (PA), and lysophosphatidic acid (LPA) are also inhibitors of GAP activity of some RGS proteins. The physiological significance for PIP_3-mediated inhibition of RGS proteins includes regulation of G protein–gated K^+ channels in cardiac myocytes [197].

2.2.3 Interactions between GPCRs and RGSs

GPCR signaling is extremely complex in mammalian cells. There are over 900 GPCRs, more than 30 RGSs and 23 different Gα proteins in mammals [210-212]. In addition, the majority of mammalian cells express multiple GPCRs, RGSs and G proteins. The mechanisms involved in the integration of the divergent signaling events remains largely unknown.

Early studies by Xu et al. indicated that different RGSs displayed differential abilities to inhibit signaling mediated by the same Gα protein that was activated by different receptors, such as muscarinic cholinergic, bombesin or cholecystokinin receptor [213]. This suggests that specific GPCRs are involved in mediating RGS effects selectively. Presently, a great deal of accumulating evidence further serves to support the notion that RGS selectivity occurs via the ability of different RGSs to specifically interact not only

with G proteins but also with different subsets of GPCRs [118,209]. For instance, Bernstein et al. [214] demonstrates that RGS2 selectively binds to the third intracellular loop (i3) of the M1 muscarinic acetylcholine receptor to specifically modulate $G\alpha_{q/11}$ signaling from this receptor through its RGS region.

The scaffold protein, spinophilin, was found to bind to both RGS2 and $G\alpha_{q/11}$-coupled $\alpha 1_B$-adrenoceptor [215]. Spinophilin also binds RGS1, 4, 16 and 19 and the i3 loops of the dopamine D_2 receptor and $\alpha 2_{A/B/C}$-adrenoceptors suggesting that there is sometimes a requirement for scaffolding proteins to bring RGS proteins and GPCRs together [177,209].

Although the trimeric receptor-G protein-RGS trimeric protein interaction model [170] gives a possible explanation for the observed RGS selectively, little is known on how RGSs selectively find and /or selectively target specific GPCRs and/or G proteins *in vivo* [216].

2.3 Yeast as a model to study mammalian GPCRs and RGSs

The yeast *Saccharomyces cerevisiae* has proven to be a useful and genetically tractable model system to study GPCR signaling [211]. It contains only 2 distinct GPCR pathways that are similar in many respects to these in mammalian cells. The first responds to pheromone stimulation and mediates the mating pathway, while the second regulates the glucose sensing pathway and invasive growth [217,218]. The *SST2* encoded

RGS serves to regulate the pheromone-response pathway in yeast. Yeast cells lacking *SST2* show prolonged GPCR mediated responses while *SST2* overexpression severely attenuates these responses to GPCR stimulation. A number of groups have shown that mammalian RGSs can functionally replace the yeast Sst2p RGS [191,203,211,219-222].

3. Prelude to my projects

Our lab has extensively used yeast to characterize the RGSs that we have identified as being expressed in the heart [203,222-224]. We have shown that mammalian RGS1, 2, 5 and 16 can replace the *SST2* yeast gene and inhibit signaling from the *STE2* encoded yeast pheromone receptor [203,223]. We have also shown that these RGSs preferentially inhibit signaling from the heterologously expressed human somatostatin receptor 5 (SST5) than from the endogenous *STE2* yeast α-factor receptor [223]. This later observation is consistent with the notion that RGS interaction with both G proteins and GPCRs serves to regulate the *in vivo* selectivity of the different RGSs [170,216].

My project was initially based on a paper that our lab published which showed that the heterologous expression of sepsis induced RGS1 leads to yeast cell growth arrest, which was dose-dependent, but independent of either of 2 endogenous yeast G proteins, Gpa1 and Gpa2 [222]. This result implies that human RGS1 expression caused cell growth suppression in yeast and either represents a novel function of RGS1 or induces a non-specific cell death. Based on this point, it is reasonable to identify RGS1 interacting proteins that may regulate RGS1 effects from screening a mammalian cDNA library.

Using RGS1 expressing yeast cells and a mouse T cell cDNA library, I successfully identified sphingomyelin synthase 1 (SMS1) cDNA sequence that was capable of suppressing RGS1 mediated cell growth arrest. Further analysis actually showed that SMS1 is not a RGS1 specific inhibitor (data not shown). I was, therefore, drawn to investigate the process of cell death in yeast. To examine cell death, I first switched from

using RGS1 to using Bax to induce cell death in yeast since it is a powerful inducer of apoptosis. Further results indicated that SMS1 prevents apoptosis in response to multiple stresses including Bax overexpression and ROS [124,225].

Sphingomyelin synthase plays a critical role in sphingolipid metabolism. It catalyzes the conversion of ceramide and phosphatidylcholine to sphingomyelin and diacylglycerol [39,226]. On the cell surface, sphingomyelin is the main component to form the barrier, which protects cells, and lipid rafts which mediate signal transductions. This SMS mediated pathway is thought to be the only means by which sphingomyelin is synthesized in cells [226-230].

Although human SMS1 and 2 have only recently been cloned [71,231], numerous studies in mammalian cells have suggested that SMS may serve an anti-apoptotic role [232-234]. During my PhD studies, I used Bax and other apoptotic stimuli to perform a series of tests. Since the pro-apoptotic Bax likely serves, at least in part, to induce death by releasing Cyt *c* from mitochondria in yeast as it does in mammalian cells, it suggested that SMS1 was an anti-apoptotic gene [235]. My data directly characterized SMS1 as an anti-apoptotic protein [124]. This part of work will be shown in Chapter 2. In Chapter 3, I also describe the alternatively spliced mouse SMS1 gene organizations [225].

Our lab nevertheless has a continuing interest in characterizing the structure and function of mammalian RGSs in yeast. When I started my PhD, our lab had just developed specific anti-sera for human RGS5. I was involved in the study that used the anti-sera to show that RGS5 protein is expressed only in cardiac and skeletal muscle [236]. We had also observed that western blot analysis suggested that RGS5 may also

exist as a dimer. As a final project for my PhD, I carried out a more detailed analysis of the possibility that RGS5 may dimerize [237]. This part of work will be presented in Chapter 4.

4. Figure Legends and Figures

Figure 1.1 Apoptotic extrinsic and intrinsic pathways (Modified from [233]).

There are two main apoptotic pathways — an extrinsic (death-receptor) pathway and an intrinsic (mitochondrial) pathway. When apoptotic stimuli stress cells, the extrinsic pathway is mediated by caspase-8 whereas the intrinsic pathway is mediated by caspase-9. FADD (FAS-associated via death domain) is an adapter protein that couples death receptors, such as CD95, to caspase-8. Cellular caspase-8 (FLICE)-like inhibitory protein (cFLIP) prevents caspase-8 activation. In certain cell types, the two pathways are interconnected by truncated BID (tBID) that is formed when BID is cleaved by active caspase-8. Bcl-2 and Bcl-x_L inhibits the loss of mitochondrial membrane potential, whereas BAX domains induce loss of mitochondrial membrane potential. Cytochrome *c* is released from the mitochondria and together with apoptotic-protease-activating factor 1 (Apaf1) and pro-caspase-9 form the apoptosome. Second mitochondria-derived activator of caspase (SMAC) is also released from the mitochondria and blocks the effect of inhibitor-of-apoptosis proteins (IAPs) to prevent caspase activation.

Figure 1.2 Structures of apoptotic caspases in mammals (Modified from [19]).

The effector and initiator caspases are shown in red and purple, respectively. The position of the first intra-chain activation cleavage (between the large and small subunits, p20 and p10, respectively) is highlighted by a black arrow, whereas other sites of cleavage are

represented by grey arrows. The prodomains in initiator caspases invariably contain homotypic interaction motifs, such as the caspase-recruitment domain (CARD) and the death-effector domain (DED). The four surface loops (L1–L4) that shape the catalytic groove are indicated. The catalytic residue Cys is shown as a red line at the beginning of L2. The p20 and p10 subunits together form a caspase monomer. The caspases and the location of functional segments are drawn to scale.

Figure 1.3 Bcl-2 family proteins [234].

The scheme shows the structure of Bcl-2 family proteins that are divided into 3 groups: anti-apoptotic members, Bcl-2 and Bcl-x_L, contain BH 1-4 domains and TM; pro-apoptotic members, Bax and Bak, contain BH 1-3 domains and TM; pro-apoptotic BH3-only consists of 2 subgroups, Bim, Bik, NIX et al. contain BH3 and TM, whereas Bid, Bad, PUMA et al. only have BH3 domain, NOXA contains 2 BH3 domains. BH, Bcl-2 homology domain; TM, transmembrane domain.

Figure 1.4 Classification of RGS proteins [118].

Sequence identities within the RGS domain establish nine mammalian subfamilies: RZ (A), R4 (B), R7 (C), R12 (D), RA (E), GEF (F), GRK (G), SNX (H) and atypical. A schematic diagram depicting the structural motifs present in a representative member (shown by a "*") of each RGS family Abbreviations used to describe the different domains are as follows: AH, Amphipathic helix; βCat, β-Catenin interacting domain; CYS, polycysteine region or cysteine string; DEP, Dishevelled domain; DH, Dbl

homology domain; DIX, Dishevelled-interacting domain; GGL, Gγ-like domain; GoLoco, $G\alpha_{i/o}$-Loco-interacting domain; GSK3β, Glycogen synthase kinase β-interacting domain; PDZ, PSD-95/Dlg/ZO-1 domain; PP2A, Phosphatase 2A-interacting domain; PTB, Phospho-tyrosine binding domain; PX, Phosphatidylinositol binding domain; PXA, Phosphatidylinositol-associated domain; RBD, Rap½- or Ras binding domain; PH, Pleckstrin homology domain; RGS, Regulator of G protein signaling domain; Ser/Thr Kinase, Serine/Threonine kinase; TM, Transmembrane domain.

Figure 1.1 Apoptotic extrinsic and intrinsic pathways

(Modified from [233]).

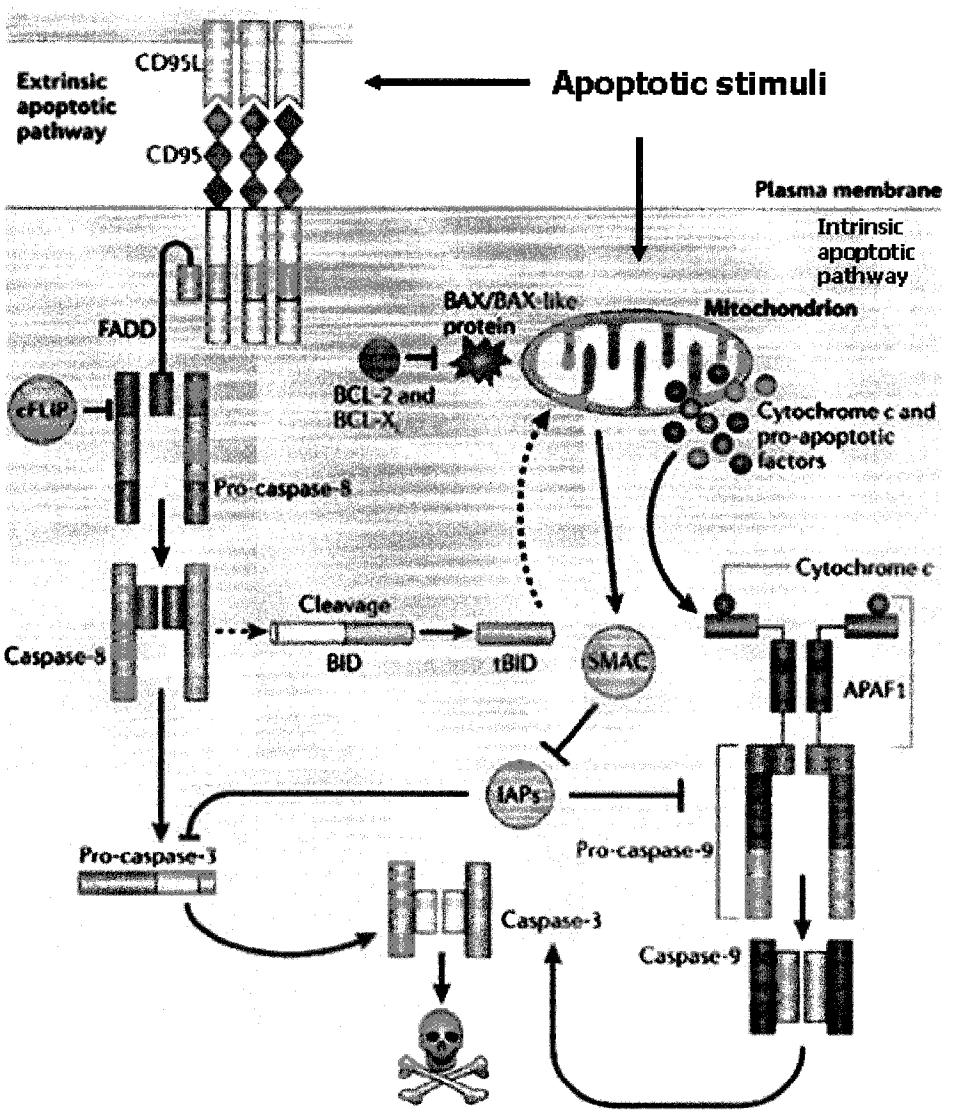

Figure 1.2 Structures of apoptotic caspases in mammals.

(Modified from [19]).

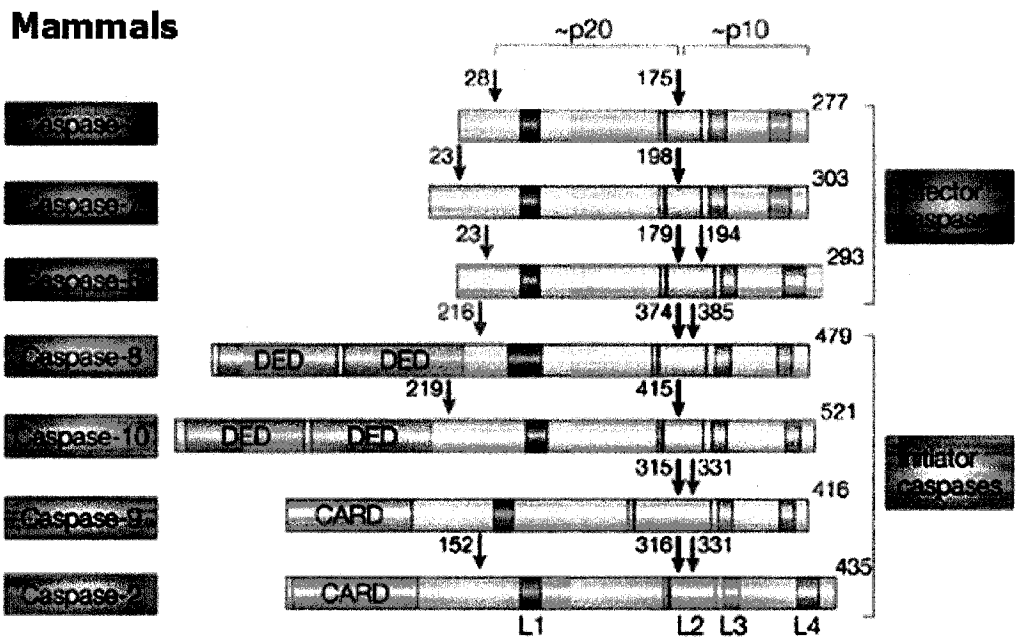

Figure 1.3 Bcl-2 family proteins [234].

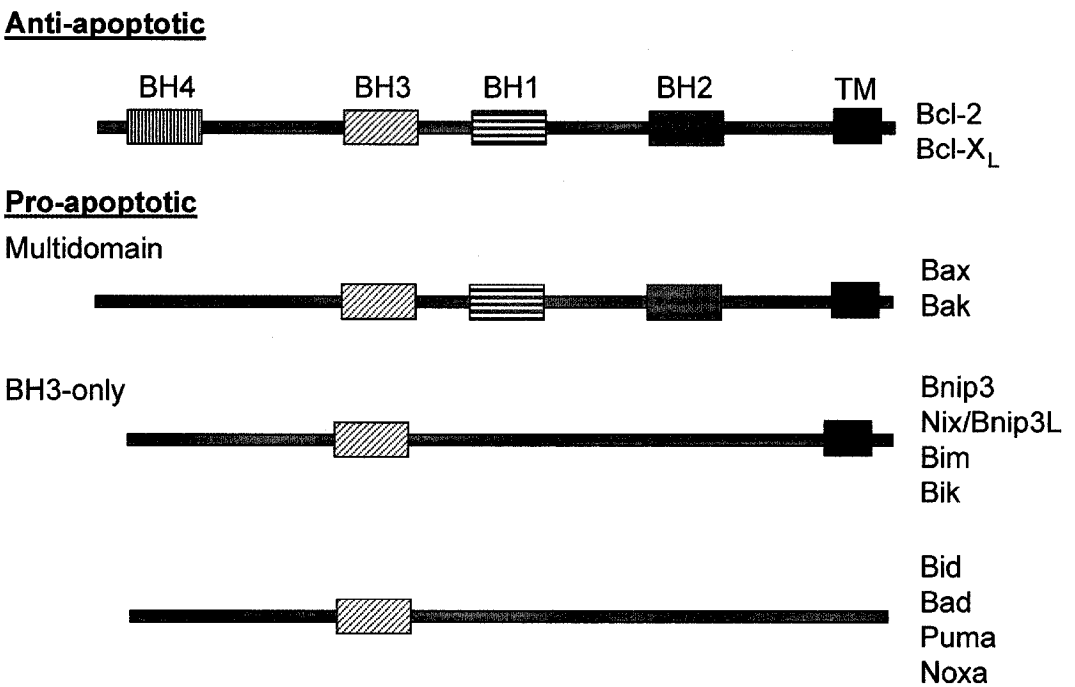

Figure 1.4 Classification of RGS proteins [118].

Family	Structure	Members
A/RZ		*RGS17 (RGSZ2), RGS19 (GAIP), RGS20 (RGSZ1), RET-RGS1
B/R4		RGS1, RGS2, RGS3, *RGS4, RGS5, RGS8, RGS13, RGS16, RGS18, RGS21
C/R7		RGS6, RGS7, RGS9, *RGS11
D/R12		RGS10, *RGS12, RGS14
E/RA		*Axin, Conductin
F/GEF		P115-RhoGEF, PDZ-RhoGEF, *LARG
G/GRK		GRK1, *GRK2, GRK3, GRK4, GRK5, GRK6, GRK7
H/SNX		*SNX13, SNX14, SNX25
Atypical		*D-AKAP2
		*RGS22

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Chapter 2

Identification and characterization of mouse sphingomyelin synthase 1 (SMS1) as a novel anti-apoptotic protein.

* This chapter is correlated with the manuscript: **Zhao Yang, Chamel Khoury, Gaël Jean-Baptiste and Michael T. Greenwood.** Identification of mouse sphingomyelin synthase 1 as a suppressor of Bax-mediated cell death in yeast. *FEMS Yeast Res.* 2006, 6(5):751-762.

1. Preface

I started my PhD project by examining RGSs in yeast. Previously, we demonstrated that human RGS1 (hRGS1) not only serves as a GAP to block GPCR signaling in yeast, but it also acts as a dose-dependent inhibitor of growth [1]. This observed inhibition led me to use those RGS1 expressing cells as a tool to screen a mouse T cell cDNA library. The strategy I used was in an attempt to identify clones that could allow cells to grow in the presence of high levels of RGS1 expression. Such clones could potentially represent novel RGS1 interacting proteins. Instead, I identified a novel anti-apoptotic clone called sphingomyelin synthase 1 (SMS1) in this screen. This leads me to start using yeast to study apoptosis.

2. Abstract

We have identified mouse sphingomyelin synthase 1 as a novel suppressor of the growth inhibitory effect of heterologously expressed human RGS1. Yeast cells expressing sphingomyelin synthase 1 were also found to show an increased resistance to a variety of cytotoxic stimuli including Bax, hydrogen peroxide, osmotic stress and elevated temperature. Sphingomyelin synthase 1 functions by catalyzing the conversion of ceramide and phosphatidylcholine to sphingomyelin and diacylglycerol. Ceramide is an antiproliferative and proapoptotic sphingolipid whose level increases in response to a variety of stresses. Consistent with its biochemical function, yeast cells expressing sphingomyelin synthase 1 have an enhanced ability to grow in media containing the cell-permeable C_2-ceramide analog as well as the ceramide precursor phytosphingosine. We also show that overexpression of *AUR1*, a potential yeast functional homolog of sphingomyelin synthase, also protects cells from osmotic stress. Taken together, these results suggest that sphingomyelin synthase 1 likely prevents cell death by counteracting stress-mediated accumulation of endogenous sphingolipids.

Key words: sphingomyelin synthase, Bax, yeast, expression cloning, cell death, cell stress.

3. Introduction

Apoptotic cell death is a genetically programmed mechanism that allows the cell to commit suicide when conditions are judged to be unfavorable [2]. The decision on whether a cell commits suicide in response to given stress is dependent on the interplay of a number of regulatory proteins. In addition to the large number of proteins that can make a cell undergo apoptosis, a number of anti-apoptotic molecules also serve to prevent apoptosis and other forms of cell death. Many of the anti-apoptotic proteins function by directly antagonizing apoptotic proteins. For example, the anti-apoptotic Bcl-2 is probably the most studied inhibitor of apoptosis. It functions in part by opposing the effects of pro-apoptotic Bcl-2 members such as Bax [3]. Many of the pro- and anti-apoptotic proteins share common domains that are easily recognizable [4]. For example, the death effector domain (DED) is present in some caspases. The anti-apoptotic function of some DED-containing proteins such as FLIPs is likely mediated by their ability to interact with DED-containing caspases. A variety of other anti-apoptotic proteins contain none of these recognizable anti-apoptotic domains. These include transcription factors and ligands for certain receptors like G-protein coupled receptor (GPCRs) [5]. Other anti-apoptotic proteins include the structurally diverse group of heat shock proteins (HSPs), a variety of anti-oxidant proteins as well as a number of other proteins of unknown function such as the GTPase GIMAP8 and the adaptor/docking-like protein called Gene 33 [6-9].

The yeast *Saccharomyces cerevisiae* has proven to be extremely useful as a genetically amenable model system to study basic cellular processes including apoptosis [10-12]. Although controversy remains as to whether or not yeast undergoes apoptosis, it is nevertheless quite clear that these cells do have a genetically programmed form of cell death. In addition, there exist a number of similarities between the observed cell death in yeast and apoptosis in mammalian cells. Yeast will undergo cell death in response to a variety of stimuli that induce apoptosis in mammalian cells, including free radicals, osmotic stress, DNA-damaging agents, starvation and aging [12-20]. As observed in mammalian cells, intracellular responses to the death-promoting stresses involve the production of reactive oxygen species (ROS) as well as the involvement of the mitochondria and possibly cytochrome *c*. Yeast also contains a number of orthologues to important mammalian apoptotic genes, including AIF (*AIF1*), caspase (*YCA1*), OMI/Htr2A (*NMA111*), DJ-1 (*HSP31*), as well as a nuclease (*TAT-D*) that is a strong candidate to be involved in DNA degradation associated with cell death [21-25]. Overexpression of the yeast apoptotic proteins can serve to initiate or enhance cell death, while yeast strains lacking any of these genes show a decreased response to a number of different death stimuli.

Although yeast does not contain Bcl-2 like proteins, the heterologous expression of proapoptotic Bax or Bak in yeast serves to induce death. Numerous studies suggest that Bax-mediated cell death in yeast is mechanistically similar to the process that occurs in mammalian cells [26,27]. Numerous groups have used inducible promoters to generate yeast cells that have conditionally lethal Bax-dependent phenotypes as a system to screen

heterologous cDNA libraries and identify novel antiapoptotic sequences. The sequences identified represent a variety of different proteins, including DNA binding proteins (HMGB1), prion protein (PrP^C), enzymes involved in inhibiting free radicals such as peroxidases, Bax-binding proteins such as BI-1 and Ku70, a plant VAMP protein involved in vesicular trafficking, as well as a variety of proteins of unknown function [6,8,28-34].

Regulators of G-protein Signaling (RGSs) negatively regulate GPCR signaling by accelerating the GTPase activity of the G_α subunit of the heterotrimeric G protein thereby serving to regulate the lifetime of the receptor activated G protein [35]. Since the initial observation that human RGS1 inhibited growth when expressed under the control of the galactose inducible *GAL1* promoter. Surprisingly this RGS1 mediated growth inhibition did not require a functional *GPA1* or *GPA2* encoded G protein [1,36]. This suggests that the growth inhibitory effects of RGS1 in yeast either represents a novel function or induces a non-specific effect due to its overexpression. In order to further characterize this RGS1 mediated effect, we screened a mouse T cell cDNA library to identify sequences capable of suppressing the growth inhibitory effect of RGS1 in yeast. Here we report that a mouse sphingomyelin synthase 1 (SMS1) cDNA suppresses the negative effect of human RGS1 expression on yeast growth. We further demonstrate that SMS1 not only suppresses the effects of RGS1 overexpression, but it also blocks the deleterious effects of well known pro-apoptotic Bax as well as a number of other stresses, including hydrogen peroxide, osmotic stress, elevated temperature and exogenously supplied sphingolipids. Given that SMS1 uses sphingolipid ceramide as a substrate to synthesize

sphingomyelin [37], our results suggest that SMS1 protects against cell death by reversing the stress-inducible increase in the levels of proapoptotic ceramide.

4. Materials and Methods

4.1 Yeast strains and plasmids

BY4741 (*MAT***a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) was used throughout this study. Plasmid p425GAL1RGS1 expressing the human RGS1 sequence under the control of the *GAL1* promoter was previously described [38]. A mouse Bax cDNA (a gift from Dr. A. LeBlanc) cloned into the *HIS3* containing vector pGILDA was used to express Bax under the control of the *GAL1* promoter. The *AUR1*-C sequence was amplified by PCR using pYC070 [39] as a template and the following forward 5'-ACTAGTGGATCCCCCGGGCTGCAGGAATTCGATTGCGTATGGCAAACCCTTTT TCG-3' and reverse 5'-CATGGTGGCGATGGATCCCGGGCCCGCGGTACCAGCCC TCTTTACACCTAGTGACGT-3' oligonucleotide primers. The *AUR1*-C PCR product was subcloned in frame with GFP using *Spe*I and *Hin*dIII into plasmid p425GAL1-GFP to make p425GAL1-*AUR1*-C-GFP [36]. The GFP-expressing plasmid p425GAL1-ATG-GFP was used as control for the expression of *AUR1*-C-GFP in yeast.

4.2 Yeast growth and transformations

Synthetic minimal media consisting of yeast nitrogen base (YNB) containing 2% glucose supplemented with the appropriate amino acids and bases was used to routinely grow yeast [40]. Unless otherwise indicated, all cells were incubated at 30°C and allowed to grow for 3 to 4 days. In order to induce expression of sequences under the control of the *GAL1* promoter, glucose was replaced with 2% galactose and 2% raffinose. The cDNA library, as well as individual plasmids, was introduced into yeast using lithium acetate. The resultant transformants were selected and maintained by omitting the appropriate nutrient from the growth media.

4.3 Screening of mammalian cDNA libraries in yeast

The mouse T-cell cDNA library was cloned into the galactose-inducible pYES2 yeast expression vector [41]. Yeast cells harboring p425GALRGS1 were transformed to uracil prototrophy using plasmid DNA from the cDNA libraries. The transformants were directly plated onto YNB nutrient agar containing galactose, and allowed to grow for 5–6 days. Cells expressing RGS1 give rise to smaller colonies. A total of *c.* 300,000 transformants from the T cell library were screened for suppressors of RGS1 by identifying colonies that have increased in size. Large colonies were purified by replating

onto fresh media plates. Plasmid DNA was subsequently isolated from the transformants that gave rise to large colonies after purification. These plasmids were then reintroduced into yeast cells harboring RGS1 and their colony size was compared to control cells expressing RGS1 alone. The nucleotide sequence of the clones that still gave rise to large colonies were then determined.

4.4 Cell growth and viability assays

The spot growth assay was used to assess the ability of different yeast transformants to grow under different conditions. Briefly, freshly saturated glucose cultures of the different transformants were serially diluted (1 to 5) and 10μl of each dilution was directly spotted on nutrient agar plates containing either glucose or galactose. In order to ensure that an equal number of cells were used, cell numbers for the different cultures were made equal in the first dilution using the spectrophotometrically (OD_{600}) measured cell densities. For all spot assays shown, identical results were obtained in at least three independent experiments. Viability was determined by microscopical examination of cells stained with the vital dye trypan blue [42]. Cells were stained with 0.1% trypan blue for 5 min and at least 300 cells were scored for each time point.

The viability of cells expressing Bax in the absence and in the presence of SMS1 was also determined by the colony formation assay. This assay was performed by using freshly saturated glucose-grown cells to inoculate galactose-containing media. The cells

were grown for 18–24h, cell number was determined microscopically, and after serial dilution 300 cells were plated on nutrient agar and incubated for 2–3 days and the resultant colonies were counted. At least three plates were scored per experiment.

For growth curves, freshly saturated cultures grown in YNB glucose-containing media were diluted into fresh galactose-containing YNB media. The cultured cells were continuously incubated at 30°C, aliquots were removed at different times and cell density was determined spectrophotometrically (OD_{600}).

The effect of ceramide on cell growth was determined essentially as described [43]. Briefly, 2ml of YNB galactose media were inoculated at low cell density (1.5×10^4 cells /ml) using freshly saturated cultures of yeast cells harboring pYES-SMS1 or the empty vector. The cultures were grown with increasing concentrations (0 to 20 μM) of the cell-permeable ceramide analog C_2-ceramide (CalBiochem, San Diego, CA). The cells were incubated overnight and growth was determined by measuring the density of the cultures at 600nm. An OD_{600} between 0.3 and 0.5 was routinely observed in the control cultures grown without ceramide.

4.5 Analysis of the effect of the RGS1 suppressors on *GAL1* promoter function

Soluble protein was extracted by heating NaOH treated yeast cells in SDS-PAGE

loading buffer for 3 min at 95°C [36,46]. An equal amount of cell extract of the different samples were separated by SDS-PAGE, transferred to PDVF membrane and sequentially challenged with anti-RGS1 antibody (Santa-Cruz Biotech) and with HRP-conjugated donkey anti-goat secondary antibody. Signals were subsequently developed using chemiluminescent luminol reagent and exposure to X-ray film.

5. Results and Discussion

5. 1 Identification of mouse SMS1 as a suppressor of human RGS1-mediated growth inhibition in yeast

We have previously demonstrated that yeast cells expressing human RGS1 under the control of the *GAL1* promoter show a galactose-dependant inhibition of growth [1]. In an attempt to identify potential RGS1 interacting proteins, we have used these cells to screen a cDNA library for sequences that will promote the growth of RGS1 expressing cells. It was reasoned that the expression of a cDNA encoding an RGS1 interacting protein would lead to enhanced growth and this could be detected by an increase in colony size.

Yeast cells harbouring plasmid p425GAL1hRGS1 were transformed with a mouse T cell cDNA library cloned in the yeast expression vector pYES2 [41]. The colony size of approximately 300,000 transformants was examined on selective galactose containing

nutrient agar plates. Under these conditions RGS1 expressing cells form very small colonies compared to non RGS1 expressing cells [1]. Twenty three transformants were initially identified as potential candidates on the basis of an increase in colony size. The colonies were further purified and six of the transformants were determined to grow faster than the controls. Plasmid DNA then isolated and re-introduced into naïve cells harbouring p425GAL1hRGS1 or the control plasmid p425GAL1 subsequently. Of those six, only one clone was found to lead to the formation of larger colonies on nutrient galactose agar plates when co-expressed with RGS1.

The complete nucleotide sequence of the RGS1 suppressor revealed a 2364 bp cDNA (GenBank accession No. AY509044). The longest open reading frame deduced from the sequence of the cDNA consisted of a 413-residue protein. This predicted 413-residue mouse protein was found to be 97% identical to the recently identified human sphingomyelin synthase 1 (SMS1) [44]. Although the SMS1 protein has been previously identified, the SMS1 clone that we have identified represents a novel cDNA, which contains a unique 5' UTR sequence that is not present in the GenBank database, suggesting that it represents an alternatively spliced variant [45].

5. 2 SMS1 prevents the growth inhibitory effects of RGS1

As a first step towards characterizing SMS1, yeast cells were transformed with plasmids p425GAL1RGS1 or pYES-SMS1 either alone or together in combination. The transformants were grown to saturation in glucose media, serially diluted (1 to 5) and

spotted onto selective nutrient agar plates containing either glucose to repress the expression or galactose to induce the expression of RGS1 and/or SMS1. No differences were observed in the growth of transformants on glucose containing media (Figure 2.1*A*, left panel), neither of control or SMS1 expressing cells on galactose media (Figure 2.1*A*, right panel). In contrast, the growth of cells harbouring p425GAL1RGS1 was inhibited when galactose presented. Co-expression of SMS1 largely abolished the growth inhibitory effects of RGS1 overexpression (Figure 2.1*A*, right panel).

It remains possible that SMS1 promotes the growth of RGS1 expressing cells by serving to inhibit the *GAL1* promoter. A SMS1-mediated repression of the galactose dependant RGS1 expression would be expected to result in an enhancement of cell growth since RGS1 is a dosage dependant inhibitor [1]. We therefore performed western blot analysis to compare the levels of RGS1 in control and SMS1 expressing cells. As seen in Figure 2.1*B*, a single band of 24 kDa closely corresponding to the calculated 22,4 kDa size of human RGS1 is only detected in cells harbouring plasmid p425GAL1RGS1 [46]. There is no observable difference in the levels of RGS1 protein in cells expressing RGS1 alone or with SMS1.

5.3 Characterization of mouse SMS1 as a suppressor of Bax-mediated growth inhibition in yeast

The result that SMS1 prevents RGS1 cell death suggests that SMS1 may be an anti-apoptotic gene. Bax is a pro-apoptotic member of the Bcl-2 family that leads to

apoptotic cell death when overexpressed in yeast [27,47]. To test the effects of SMS1 on Bax-mediated cell death, yeast cells were transformed with the plasmids pGILDA-Bax or pYES-SMS1, either alone or in combination. The transformants were grown overnight in liquid glucose-containing media and serial dilutions were spotted onto glucose- and galactose-containing nutrient agar plates. Since both the SMS1 and Bax cDNAs are expressed under the control of the yeast *GAL1* promoter, there was no difference in the growth of the different transformants on glucose-containing plates (Figure 2.2A, left panel). In contrast, yeast cells containing the galactose-inducible mouse Bax cDNA failed to grow to any significant extent on galactose-containing plates, when compared to yeast cells that contain control plasmids (Figure 2.2A, right panel). Overexpression of SMS1 alone had no detectable effect on growth, while cells co-expressing SMS1 and Bax showed a significant increase in growth (Figure 2.2A, right panel).

We also used growth curves to monitor the effect of SMS1 on the growth of Bax-expressing yeast cells. Glucose-grown cells were inoculated into fresh galactose media and the growth of the cells was subsequently monitored over time at 30°C. Yeast cells expressing Bax alone showed a significant decrease in the rate of growth and the final cell density reached, when compared to control cells harboring empty vectors (Figure 2.2B). The expression of SMS1 was found to enhance both the growth rate and the final culture density of cells expressing Bax.

To examine the possibility that SMS1 may also be preventing Bax-mediated cell death, we examined the viability of yeast cells expressing different plasmids. Freshly saturated glucose-grown cultures of the different yeast transformants were diluted into galactose-containing media and allowed to grow for 6h. Aliquots of the cultures were

removed and cell viability was then determined by microscopical examination of cells stained with the vital dye trypan blue. Viable cells exclude the trypan blue, so the ratio of blue to colorless cells was used to determine the percentage of viable cells. Yeast cells containing empty vectors or the SMS1-expressing plasmid showed greater than 90% viability in this assay (Figure 2.2C). In contrast, the viability of Bax-expressing cells was reduced to 61.8%. Yeast cells coexpressing SMS1 along with Bax showed an increase in viability up to 74.3%.

We also used the colony formation assay to determine the ability of SMS1 to prevent Bax-mediated cell death. In this assay we found that only 19% of Bax-expressing cells were able to form colonies. The viability of Bax-expressing cells was increased to 80% when SMS1 was co-expressed (Figure 2.2D). The observed differences in viability of Bax-expressing cells in the two assays reflects the differences in time that Bax was expressed prior to determining viability and the fact that cells can exclude vital dye and appear alive, yet are unable to form colonies when plated. This can lead to an overestimation of the number of viable cells [48].

These results indicate that SMS1 can partially prevent Bax-mediated cell death. The identification of SMS1 is to our knowledge, the first case in which an enzyme involved in sphingolipid metabolism has been identified as an inhibitor of the apoptotic effects of Bax. Nevertheless, these results are consistent with numerous other studies that have implicated sphingolipid-metabolizing enzymes, including SMS, as being antiapoptotic in mammalian cells [48-50].

5.4 SMS1 decreases cell death in response to hydrogen peroxide and high osmolarity

Although Bax expression in yeast clearly leads to cell death, some controversies nevertheless remain as to whether this process truly mimics apoptosis or some other form of programmed cell death, such as autophagy [27]. We therefore ascertained whether SMS1 prevents cell death in response to other stimuli. ROS are common byproducts of metabolism, causing cellular damage that can also induce apoptosis. A number of chemicals, such as hydrogen peroxide, are used as ROS donors to induce cell death in yeast cells [19].

To test the effects of SMS1 on H_2O_2-mediated cell death, glucose-grown cultures of cells harboring either control or SMS1-expressing plasmid were incubated in galactose media for 5h and challenged with increasing concentrations of hydrogen peroxide for a further 5h. A 10μl aliquot of each culture was then spotted onto nutrient agar media containing either glucose or galactose and allowed to grow at 30°C. Compared to the controls, cells expressing SMS1 showed enhanced ability to grow, already evident after being treated with as little as 5mM H_2O_2 (Figure 2.3A). Cells expressing SMS1 during the period of H_2O_2 treatment showed enhanced growth if plated in the presence of glucose or galactose, indicating that the expression of SMS1 was not required for the cells to recover after H_2O_2 treatment. Thus SMS1 is required to protect the cells from H_2O_2 treatment and it is not required for growth after the stress is over.

The vital dye, trypan blue, was used to ascertain the effect of SMS1 on cell viability

after H_2O_2 treatment. As shown in Figure 2.3B, control yeast cells showed a dose-dependent decrease in viability with increasing concentrations of H_2O_2. The negative effect of H_2O_2 on cell viability was largely abolished in cells expressing SMS1. Viability was decreased to 47% in control cells treated with 20mM H_2O_2, while the viability of cells expressing SMS1 remained at 75% of untreated control cells (Figure 2.3B).

Osmotic stress leads to growth inhibition and it can also induce cell death via apoptosis [17]. We therefore examined the ability of SMS1 to prevent the growth inhibitory effects of high salt. Freshly saturated glucose-grown cultures of control as well as of SMS1-containing cells were serially diluted and aliquots spotted onto nutrient media agar plates containing glucose or galactose and 6% NaCl. On galactose medium, SMS1-expressing cells showed an enhanced ability to grow on media containing 6% NaCl compared to control cells (Figure 2.4A, right panel). Growth of both cells was identical on glucose media containing 6% NaCl, indicating that the same numbers of control and SMS1-expressing cells were used in the experiment (Figure 2.4A, left panel).

We also used trypan blue staining to monitor the effect of SMS1 on the viability of cells growing on media containing 6% NaCl. The viability of control yeast cells growing on media containing high levels of salt gradually decreased from 81% after 1 day to 46% after 3 days (Figure 2.4B). In contrast, the viability of SMS1 cells showed a modest 8% decrease after 1 day of growth on high-salt media. Even after 3 days on high-salt media, the viability of SMS1 expressing cells remained high at 75% (Figure 2.4B).

The results presented so far indicate that SMS1 is capable of suppressing the apoptotic effects of a number of different stimuli. DNA-damaging agents, such as

ultraviolet light (UV), is another common stimulus leading to cell death that may involve apoptosis [51]. In the absence of UV, there was no difference in the ability of control or SMS1-containing cells to grow on glucose or galactose media (Figure 2.4C). Although there was a noticeable decrease in the ability of cells to grow after treatment with 10 mJ/cm^2 UV, there was no difference in growth between cells expressing SMS1 and control cells. Similarly, SMS1 did not protect cells from lower or higher doses of UV (5 and 50 mJ/cm^2; not shown). These results indicate that SMS1 is not able to prevent cell death in response to all stresses.

5.5 SMS1 partially reverses the growth-inhibitory effects of exogenously supplied sphingolipids

SMS is a key enzyme in sphingolipid metabolism that utilizes ceramide and phosphatidylcholine (PC) to produce sphingomyelin (SM) and diacylglycerol (DAG) [37,44,51]. Ceramide is an important sphingolipid that has been widely reported to be proapoptotic in mammalian cells and to inhibit yeast cell growth [43,52,53]. In mammalian cells, the activation of SMS has been shown to prevent apoptosis [50]. The antiapoptotic properties of SMS are likely due to its ability to utilize ceramide as a substrate.

An increase in the production of ROS leading to cellular damage such as lipid peroxidation has been implicated as being at least partly responsible for yeast cell death

in response to stressful stimuli as diverse as Bax expression and H_2O_2, as well as high osmolarity [17,19]. Since SMS1 is capable of preventing cell death in response to these stresses, it remains possible that it also functions to promote survival by reducing ROS levels in yeast. Given its known function in mammalian cells, the ability of overexpressed SMS1 to utilize ceramide is nevertheless the most likely explanation for the observed anti-apoptotic properties of SMS1 in yeast. If this is true, it therefore makes sense that yeast cells expressing SMS1 would show an enhanced resistance to the cytotoxic effects of exogenously supplied ceramide or sphingolipids that can be converted to ceramide.

To test this possibility we examined the ability of yeast cells expressing SMS1 to grow in the presence of exogenously supplied cell-permeable C_2-ceramide. Freshly saturated glucose-grown yeast cultures were used to inoculate galactose-containing media at low cell density. The cultures were grown overnight in the absence or in the presence of increasing concentrations of C_2-ceramide. As previously reported [43], ceramide caused a dose-dependent inhibition of the growth of control cells (Figure 2.5A). Although ceramide also inhibited the growth of SMS1-expressing cells, the effect was more pronounced in control cells. In the presence of 2.5μM ceramide, control cells grew only 35% as much as the untreated cells. In contrast, cells expressing SMS1 grew 76% as much as the untreated cells in the presence of 2.5μM ceramide. SMS1-expressing cells also showed more growth than control cells in the presence of both 5 and 20μM ceramide. The effect was less pronounced at 20μM ceramide, possibly indicating that there is a limit to the amount of ceramide that can be enzymatically converted by SMS1. Nevertheless, these results suggest that SMS1 can protect cells from the growth-inhibitory effects of

exogenous ceramide.

In yeast, phytosphingosine (PHS) is a sphingolipid intermediate that is converted to phytoceramide by the action of phytoceramide synthase [37,54]. Phytoceramide is then converted to inositol phosphorylceramide (IPC) by the action of the *AUR1*-encoded IPC synthase. Yeast cells lacking *AUR1* are not viable and accumulate high levels of phytoceramide when given PHS [55]. In addition, exogenously supplied PHS is cytotoxic [56]. This suggests that Aur1p may function to rid the cell of unwanted and cytotoxic ceramides [55]. Given that the biochemical function of SMS1 is analogous to that of Aur1p in that both enzymes can use ceramide as a substrate [57], we tested the possibility that SMS1 would prevent the growth-inhibitory effects of exogenously supplied PHS. Glucose-grown yeast cells were serially diluted and spotted onto galactose-containing nutrient agar plates with or without 20μg/ml PHS. Both the control and SMS1-containing yeast cells showed equal growth on plates containing no PHS (Figure 2.5B). In contrast, yeast cells expressing SMS1 showed significantly more growth than control cells when grown in the presence of PHS. These results suggest that SMS1 is capable of promoting growth and preventing cell death in response to exogenously supplied sphingolipids that can be converted to phytoceramide. These results support the notion that SMS1 is anti-apoptotic by preventing stress-mediated increases in the levels of cytotoxic sphingolipids.

5.6 SMS1-expressing cells show enhanced resistance to chronic heat stress

In mammalian cells, an increase in ceramide occurs in response to numerous proapoptotic stimuli including heat shock [58]. Sphingolipid metabolism as well as the enzymes involved in its synthesis and degradation are also important for heat shock responses in yeast [54,58,59]. The critical role of sphingolipids is demonstrated by the observation that yeast mutants that are unable to synthesize sphingolipids are unable to grow at elevated temperatures [60]. In the early stages of heat shock or stress, the levels of complex sphingolipids such as dihydrosphingosine (DHS) and PHS are transiently elevated. More prolonged heat stress eventually leads to the accumulation of different sphingolipids, including ceramide, the roles of which are largely unknown [58].

Our results indicate that SMS1 can protect yeast cells from the growth-inhibitory and apoptotic effects of chronic stresses. Given that the only known function of SMS1 is to synthesize sphingomyelin and DAG using ceramide as a substrate. All these results suggest that SMS1 protects yeast cells by decreasing stress-mediated increases in ceramide levels. Although stress-mediated increases in ceramide leading to growth inhibition and cell death have been observed in mammalian cells, it is not commonly reported in yeast [58,61].

The observation that SMS1 prevents the cytotoxic effects of exogenously supplied ceramide and PHS in yeast supports the idea that increased ceramide levels occur in response to multiple stresses in yeast (Figure 2.5). Given that the long-term role of the

observed elevation of sphingolipids and ceramide in response to heat stress is largely unknown, we used a spot assay to examine the ability of control and SMS1-expressing yeast cells to grow at different temperatures. Freshly saturated glucose-grown cultures of cells harboring the control plasmid as well as pYES-SMS1 were serially diluted and plated onto nutrient agar media containing either glucose or galactose. The plates were subsequently incubated at 30, 37 or 41°C for 3 days. Compared to cells grown at 30°C, the control cells showed a reduction in growth at 37°C which was more pronounced at 41°C (Figure 2.6). In contrast, SMS1-expressing cells showed more growth than control cells at 37°C, and this effect was even more pronounced when cells were grown at 41°C. These results demonstrate that SMS1 promotes the growth of cells in response to the chronic stress resulting from elevated temperature. This suggests that yeast cells, like mammalian cells, also respond to chronic heat stress by increasing the levels of ceramide.

5.7 Overexpression of the yeast AUR1-C also prevents the effects of high osmolarity

The yeast Aur1p is an inositol phosphorylceramide synthase that has similar biochemical properties as the mammalian SMS1 and possibly functional similarities [57]. If SMS1 prevents stress-induced cell death in yeast, we reasoned that *AUR1* should also do the same. In order to analyze this possible role for *AUR1* in yeast, we cloned the aureobasidin A (AbA)-resistant *AUR1*-C *AUR1* mutant as a GFP fusion under the control of the galactose-inducible *GAL1* promoter into p425GAL1-GFP. Spot analysis of yeast

cells harboring plasmid p425GAL1-*AUR1*-C-GFP on YNB selective media containing glucose, glucose and AbA, or galactose and AbA, suggests that the galactose-mediated expression of *AUR1*-C-GFP leads to AbA resistance in yeast (Figure 2.7A).

Yeast cells harboring either the control GFP-expressing plasmid p425GAL1-ATG-GFP or the *AUR1*-C-GFP-expressing plasmid were grown on glucose- or galactose-containing media with 6% NaCl. Cells expressing *AUR1*-C-GFP showed enhanced growth in the presence of high levels of salt when compared to control cells (Figure 2.7B). The viability of yeast cells grown in high levels of salt for 24h was also enhanced by the expression of *AUR1*-C-GFP (90.3 ± 1.6% for *AUR1*-C-GFP expressing cells vs. 81.2 ± 1.3%, $n = 3$, for cells expressing only GFP). Although the effect was not as pronounced as with SMS1, the expression of *AUR1*-C-GFP also increased the ability of yeast cells to grow at elevated temperatures (not shown).

5.8 Summary

In summary, we have identified mouse SMS1 as a suppressor of both the growth-inhibitory and cell death-inducing effects of a mammalian Bax in yeast. In addition, SMS1 was found to prevent the inhibitory effects of other stresses, including hydrogen peroxide, high levels of salt and elevated temperature. Consistent with its known biochemical function, SMS1 was also found to confer growth resistance to exogenously supplied ceramide or to the ceramide precursor phytosphingosine. The antiapoptotic role described for SMS1 in yeast suggests that this enzyme is at least in part responsible for

the known anti-apoptotic role of SMS in mammalian cells. The enhanced ability of yeast cells that overexpress the *AUR1* gene to grow and survive in media containing high levels of salt also supports the role of inositol phosphorylceramide synthase as being anti-apoptotic. These results emphasize the usefulness of yeast as a model system to study the involvement of sphingolipids in the process of programmed cell death [37,57].

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7. Figure Legends and Figures

Figure 2.1 SMS1 suppresses the growth inhibitory effects of RGS1.

Yeast transformants that contain empty p425GAL1 and pYES2 vectors (CONTROL) are compared to cells harboring p425GAL1 and pYES2-SMS1 (SMS1), pYES2 and p425GAL1RGS1 (RGS1), or pYES2-SMS1 and p425GAL1RGS1 (RGS1+SMS1). **A.** Yeast cells were grown to saturation in YNB glucose containing media, serially diluted and 10μl of each dilution were spotted onto selective nutrient agar plates containing either glucose (GLU) or galactose (GAL) to induce RGS1 and SMS1 expression. Photographs of the resultant plates are shown after 3 days of incubation at 30°C. **B.** Levels of RGS1 protein in different transformants were determined in galactose-grown cells by western blot analysis using an RGS1 specific antibody. RGS1 was detected only in cells harbouring p425GAL1RGS1 (RGS1 and RGS1+SMS1). Molecular weight (in kDa) is shown on the left.

Figure 2.2 SMS1 suppresses the growth-inhibitory and death-inducing effects of expressing murine Bax in yeast.

The growth and viability of different yeast transformants harboring empty vector (CONTROL), Bax or SMS1-expressing plasmids were analyzed. The transformants were first grown to saturation in glucose-containing media. **A.** The saturated cultures were serially diluted and 10μl aliquots of each dilution were spotted on glucose (GLU)- and

galactose (GAL)-containing nutrient agar media. Photographs of the plates after incubation at 30°C are shown. **B.** Glucose-grown cells were used to inoculate galactose-containing media and growth was monitored by removing samples and measuring the turbidity at optical density of 600nm (OD_{600}). The following symbols denote the different strains examined: empty vector control (•), SMS1 (○), Bax and SMS1 (▵) and Bax alone (▾). **C.** Glucose-grown cultures were used to inoculate galactose-containing media. The cultures were allowed to grow for 6h and cell viability was determined by microscopical examination of cells stained with the vital dye trypan blue. At least 300 cells were scored for each data point. Data represent the mean±standard error of the mean (SEM) of three independent experiments. *,**: One-way anova analysis followed by an all pairwise comparison procedure (Holm–Sidak method) showed that there are significant differences with the control and SMS1 groups ($P<0.003$). **D.** Glucose-grown cultures were used to inoculate galactose-containing media. Yeast cells were then grown overnight to saturation in galactose-containing media and viable cells were determined as the percentage of cells that formed colonies on agar media. The viable cell number obtained from control cells harboring empty vectors was arbitrarily used as 100%. Data represent the mean ± standard deviation (SD) of triplicate assays and are typical of two independent experiments.

Figure 2.3 SMS1-expressing yeast cells are resistant to hydrogen peroxide-mediated death.

Yeast transformants harboring empty vector (CONTROL) or pYES-SMS1 (SMS1) were

grown in galactose-containing media for 5h. Identical cultures were treated with the indicated concentrations of hydrogen peroxide (H_2O_2) for 5h. **A.** 10μL of each culture were then directly spotted onto glucose (GLU)- or galactose (GAL)-containing media and photographs of the resultant plates after incubation at 30°C are shown; **B.** Viability of the H_2O_2-treated transformants harboring empty vector (black) or pYES-SMS1 (gray) was determined by microscopical examination of trypan blue-stained cells. At least 300 cells were examined for each data point and similar results were obtained in two independent experiments.

Figure 2.4 The effect of high levels of salt and UV on the growth of SMS1-expressing yeast cells.

A. Freshly saturated glucose-grown cells were serially diluted and 10μL aliquots were spotted onto glucose (GLU) and galactose (GAL) nutrient agar media containing 6% NaCl. A photograph of the plates after incubation at 30°C is shown; **B.** Cultures of freshly saturated control (black) or SMS1 (gray)-expressing yeast transformants were used to inoculate fresh galactose media containing 6% NaCl. Aliquots of cells were taken at 24h intervals and viability was determined by microscopical analysis of trypan blue-stained cells. The data are expressed as the mean percentage ± SD of viable cells and are typical of two identical experiments. **C.** Aliquots of serially diluted control and SMS1-containing cells were spotted onto nutrient agar GLU- and GAL-containing media. The control plates received no further treatment, while the other plates were illuminated with 10 mJ/cm^2 of ultraviolet light. The cells were allowed to grow at 30°C and photographs

of the plates are shown.

Figure 2.5 SMS1 reverses the growth-inhibitory effects of exogenously supplied sphingolipids in yeast.

A. Cultures of freshly saturated cultures of control yeast cells (black) or SMS1 (gray)-expressing yeast cells were used to inoculate fresh galactose-containing YNB media at low cell density. The cultures received no addition (0) or increasing amounts of C_2-ceramide (2.5, 5 or 20μM). The cultures were grown for 18–20h at 30°C and the turbidity of the cultures was determined at 600nm. Growth of the different cultures is shown as a percentage of the growth seen in the control cultures. The data are mean ± SD of triplicate cultures and are typical of identical experiments. **B.** Freshly saturated glucose-grown cells were serially diluted and spotted onto galactose-containing nutrient agar media with 0.05% Nonidet P-40 in the presence and absence of 20μM phytosphingosine. The cells were allowed to grow for 4–5 days and a photograph of the resultant plates is shown.

Figure 2.6 The effect of temperature on the growth of SMS1-expressing cells.

Freshly saturated glucose-grown cells were serially diluted and 10μL aliquots were spotted onto glucose (GLU)- and galactose (GAL)-containing nutrient agar and incubated for 4–6 days at the indicated temperatures. Photographs of the resultant plates are shown.

Figure 2.7 The yeast *AUR1*-C protects against high-osmolarity-induced growth inhibition.

The growth of yeast cells harboring the GFP-expressing plasmid p425ATG-GFP (CONTROL) or the AUR1-C-GFP-expressing plasmid (AUR1-C) was analyzed using the spot growth assay. A. Cells were plated on YNB selective media containing glucose (GLU), GLU and 0.075μg/mL of Aureobasidin A (GLU+AbA), or galactose and Aureobasidin A (GAL+AbA); **B.** Yeast cells harboring either the control or the *AUR1*-C-GFP-expressing plasmids were spotted on GLU-containing media or GAL media containing 1M NaCl (GAL+NaCl).

Figure 2.1 SMS1 suppresses the growth inhibitory effects of RGS1.

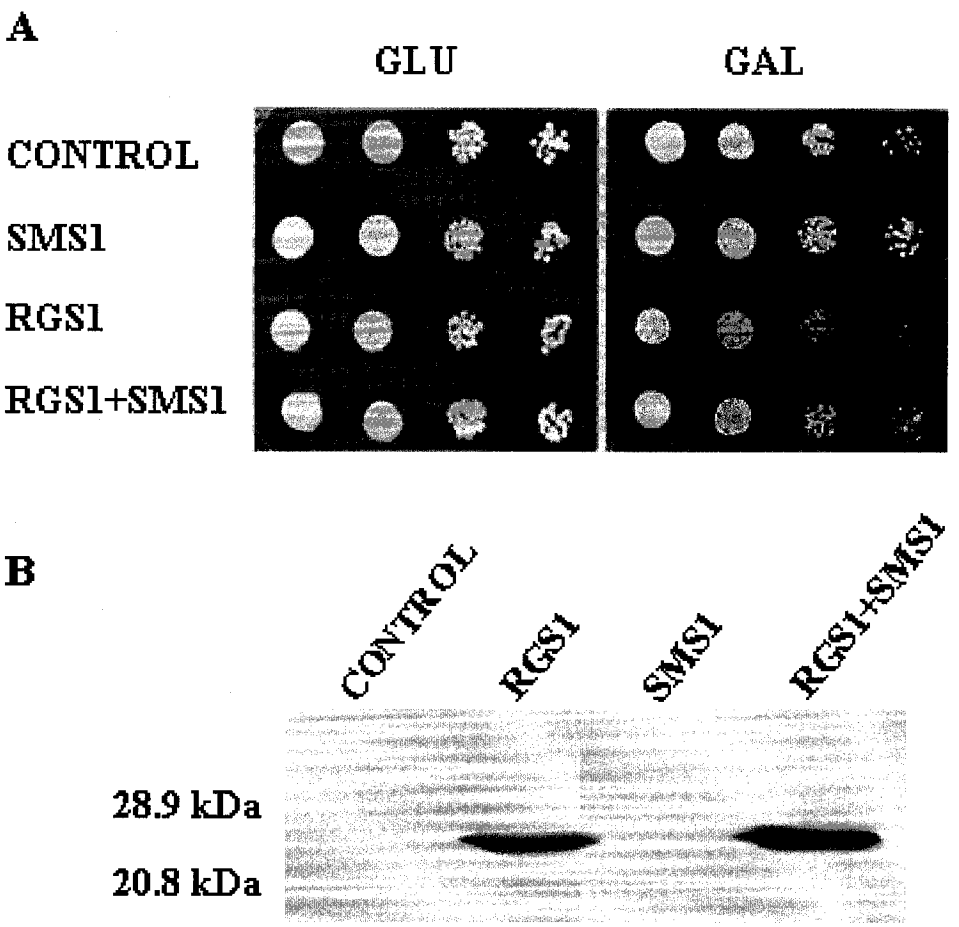


Figure 2.2 SMS1 suppresses the growth-inhibitory and death-inducing effects of expressing murine Bax in yeast.

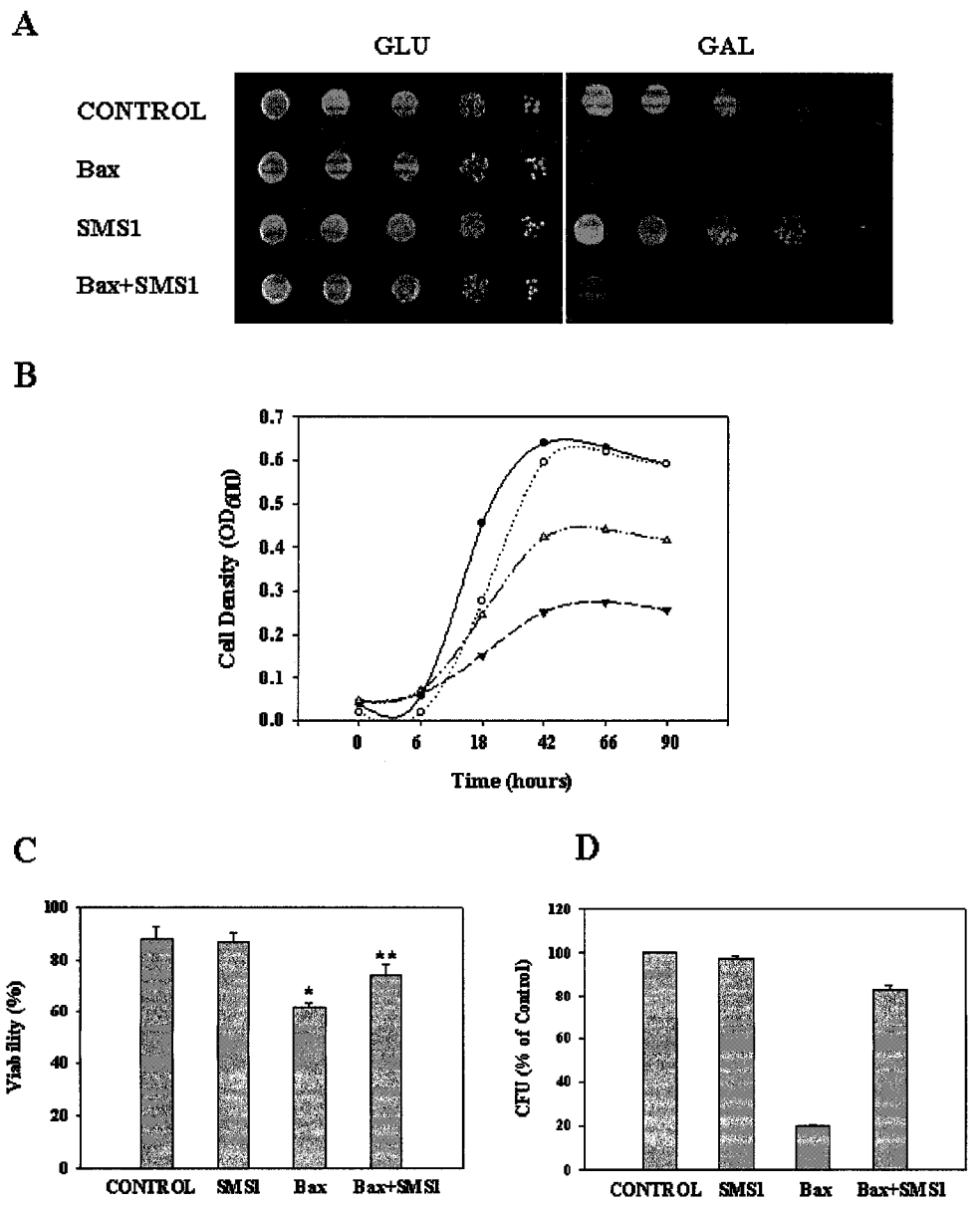


Figure 2.3 SMS1-expressing yeast cells are resistant to hydrogen peroxide-mediated death.

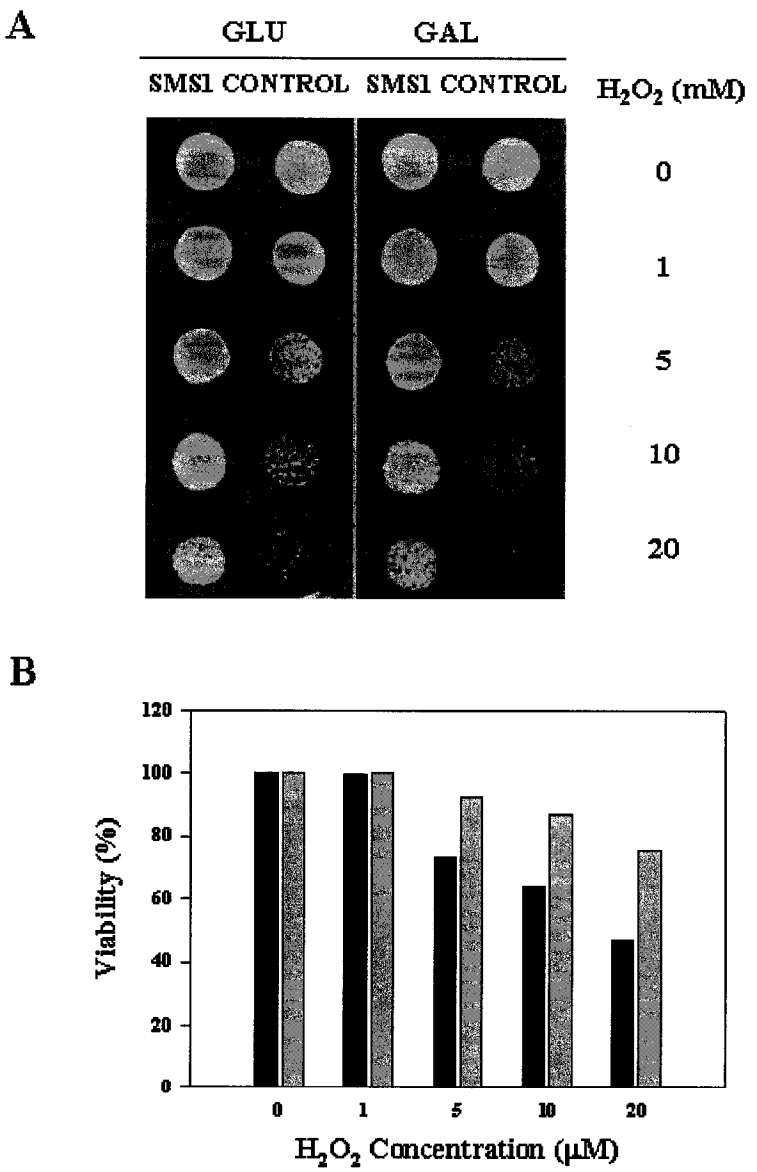


Figure 2.4 The effect of high levels of salt and UV on the growth of SMS1-expressing yeast cells.

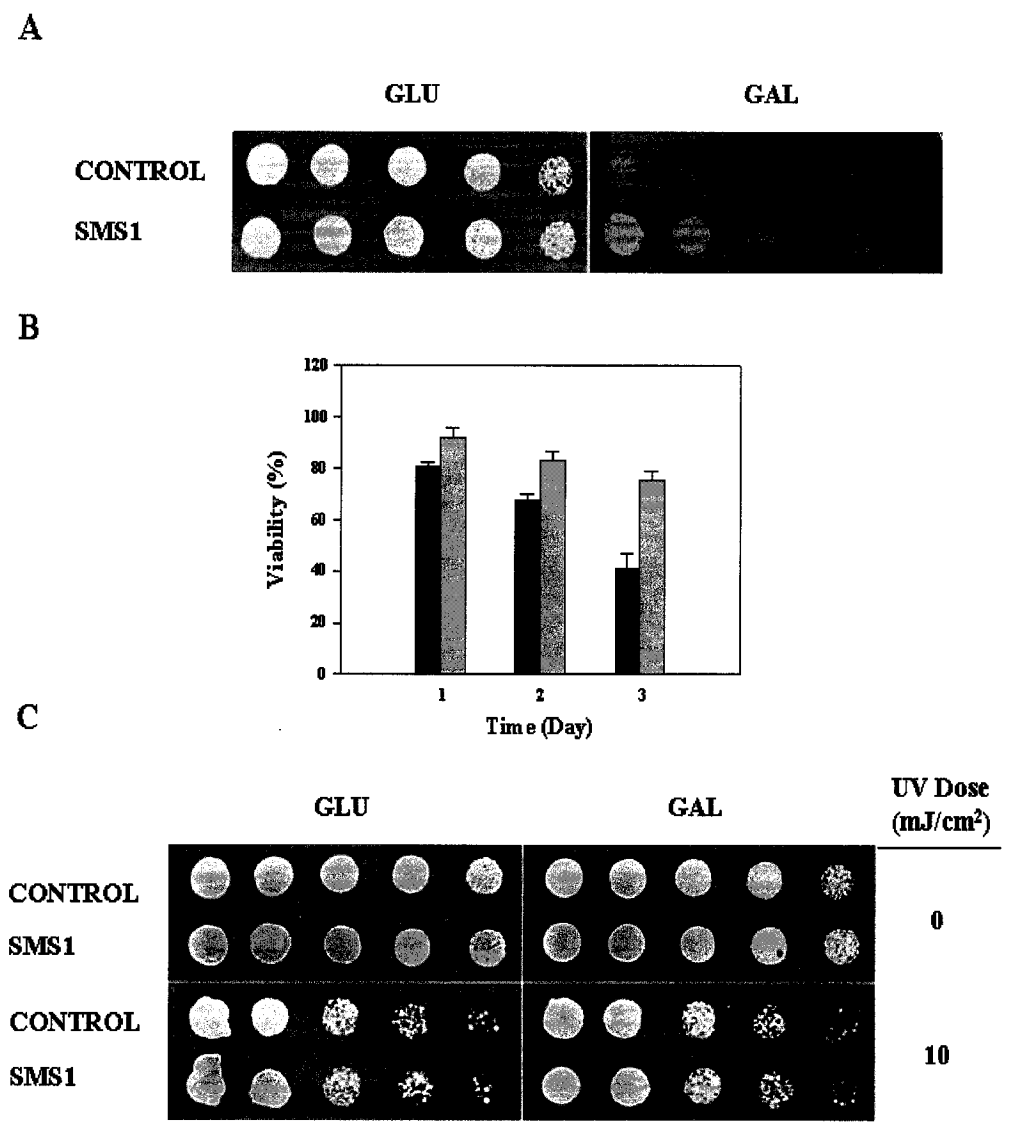
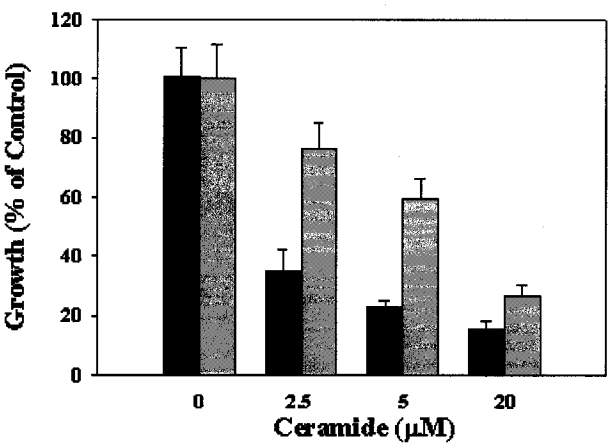


Figure 2.5 SMS1 reverses the growth-inhibitory effects of exogenously supplied sphingolipids in yeast.

A



B

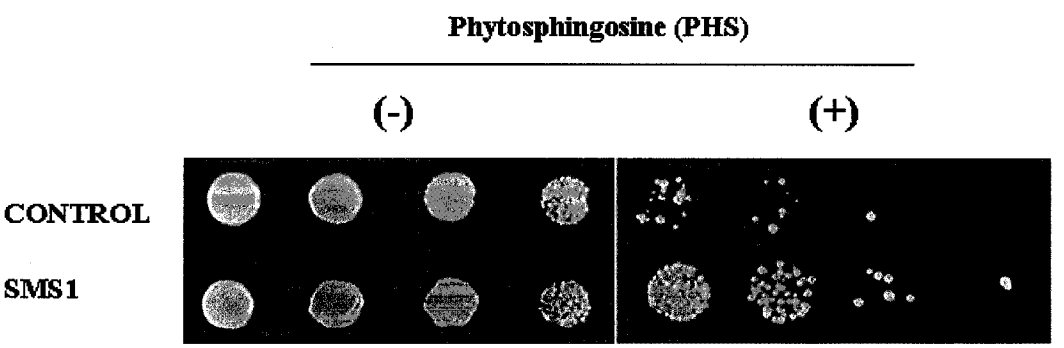


Figure 2.6 The effect of temperature on the growth of SMS1-expressing cells.

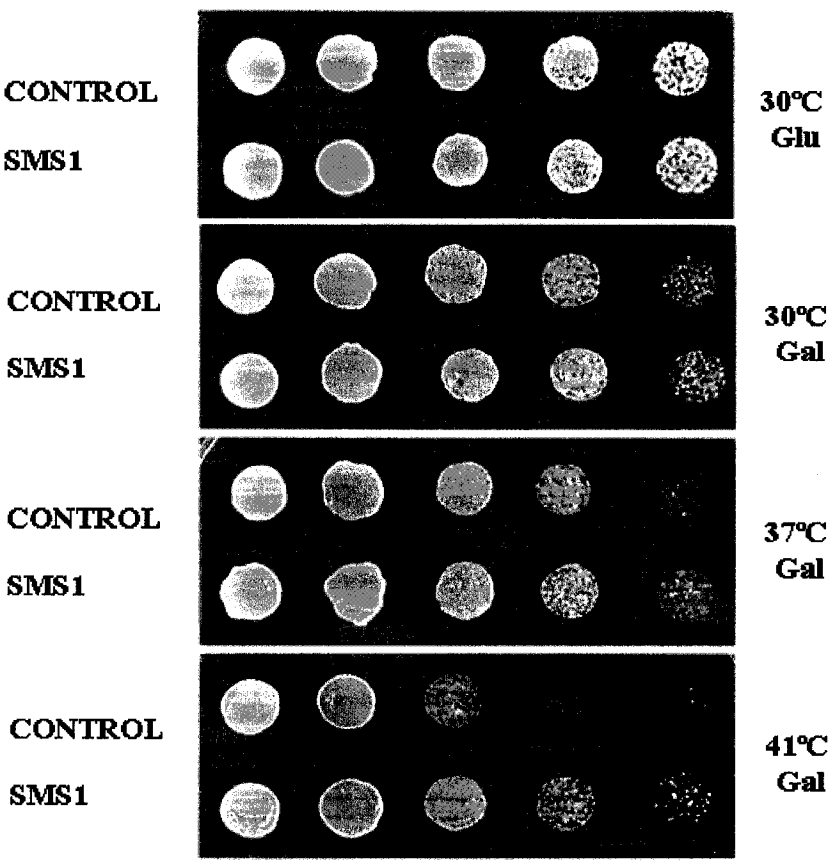
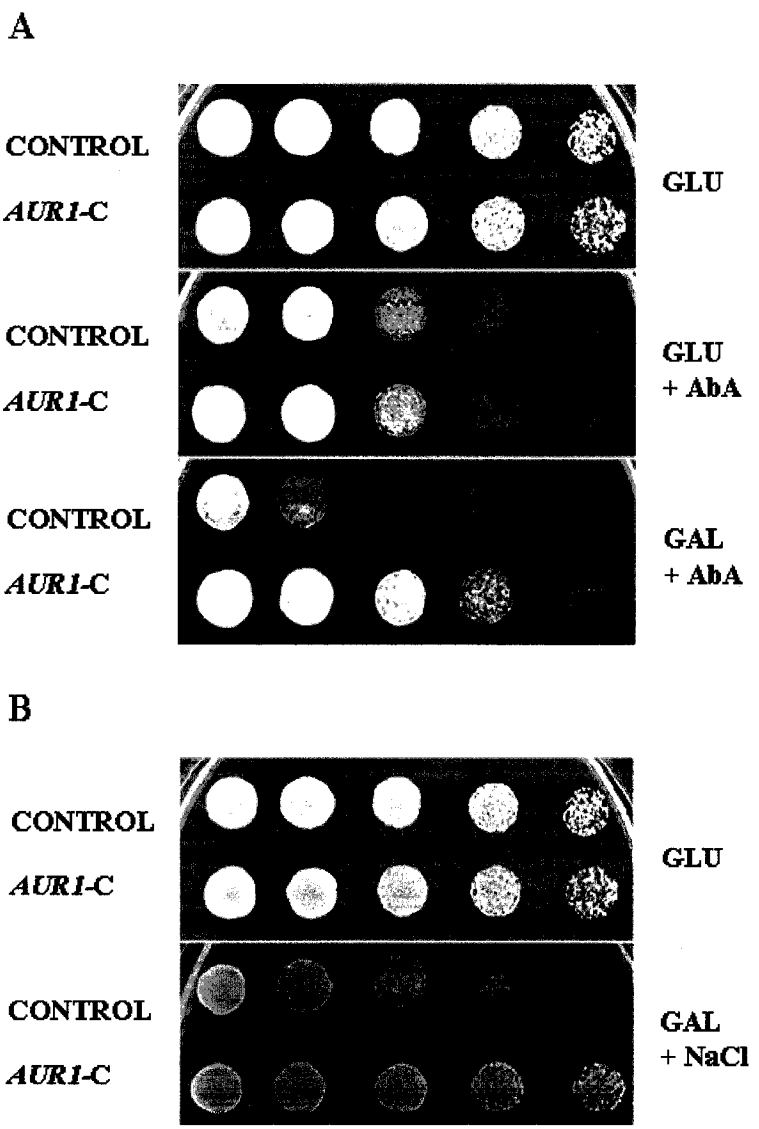


Figure 2.7 The yeast *AUR1*-C protects against high-osmolarity-induced growth inhibition.



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Chapter 3

The mouse Sphingomyelin Synthase 1 (SMS1) gene is alternatively spliced to yield multiple transcripts and proteins.

* This chapter is correlated with the manuscript: **Zhao Yang, Gaël Jean-Baptiste, Chamel Khoury and Michael T. Greenwood.** The mouse sphingomyelin synthase 1 (SMS1) gene is alternatively spliced to yield multiple transcripts and proteins. *Gene*. 2005, 363:123-132.

1. Preface

Sphingomyelin synthase (SMS) is recognized for using ceramide and phosphatidylcholine as substrates to form sphingomyelin and diacylglycerol [1]. However, the sequence of SMS has been revealed only in recent years. Luberto et al. reported SMS sequence from *Pseudomonas aeruginosa* in 2003 [2]. The human SMS sequence was published until 2004 [3,4]. I, nevertheless, contributed the mouse SMS cDNA sequence in 2005 [5,6].

Although Huitema et al. pointed out that human, mouse and *Caenorhabditis elegans* genomes each contain at least two different SM synthase (SMS) genes, SMS1 and SMS2, and indicated that human SMS1 is localised to the Golgi, whereas SMS2 resides primarily at the plasma membrane [3]. These studies provide no details regarding the structure of the SMS1 gene. The following manuscript fills in this blank.

2. Abstract

Sphingomyelin synthase 1 (SMS1) is a recently identified 413-residue protein that plays a critical role in sphingolipid metabolism by catalyzing the conversion of ceramide and phosphatidylcholine to sphingomyelin and diacylglycerol (DAG). We have previously reported the isolation of a mouse *SMS1* encoding cDNA that contains a unique 5' *UTR* sequence. Three other mouse *SMS1* cDNAs that differed in their 5' and 3' non-coding sequences were present in GenBank. In order to ascertain the origin of the unique 5' and 3' *UTR* sequences, we analyzed the structure of the mouse *SMS1* gene. Analysis of the four different *SMS1* cDNA sequences and of the corresponding mouse genomic fragment revealed that the *SMS1* gene consists of 16 exons that are alternatively spliced to produce 4 different mRNAs (*SMS1*α1, *SMS1*α2, *SMS1*β and *SMS1*γ) and 3 different proteins (SMS1α, SMS1β and SMS1γ). RT-PCR was used to demonstrate that all four *SMS1* cDNAs represent expressed transcripts that show distinctly different tissue distributions. Transcripts for *SMS1*α1, *SMS1*α2 and *SMS1*β were found to increase in response to the pro-apoptotic effects of TNF-α. Finally, using the yeast based assay, we confirmed that SMS1α prevents the growth inhibitory effects of Bax but SMS1β neither prevents nor enhances the effects of Bax or of SMS1α. Taken together these results demonstrate the complexity of SMS1 gene structure, expression and function.

Key words: Sphingomyelin synthase, Gene structure, Alternative splicing, Yeast, C2C12 cells

3. Introduction

Sphingomyelin synthase (SMS) catalyzes the conversion of ceramide and phosphatidylcholine to sphingomyelin and diacylglycerol (DAG) [7,8]. Ceramide is an important sphingolipid that has been widely reported to be pro-apoptotic in mammalian cells and to inhibit yeast cell growth [9-12]. In mammalian cells, activation of SMS prevents apoptosis [8,13]. The anti-apoptotic properties of SMS are likely due to its ability to utilize ceramide as a substrate and/or increase the levels of mitogenic DAG. Despite its importance in sphingolipid metabolism, cDNAs encoding SMSs have only been identified recently [3,4].

We have previously demonstrated that yeast cells expressing human Regulator of G-protein Signaling 1 (*RGS1*) under the control of the *GAL1* promoter show a galactose dependant inhibition of growth [14]. In an attempt to identify potential RGS1 interacting proteins, we have used these cells to screen a mouse T cell cDNA yeast expression library for sequences that will promote the growth of *RGS1* expressing cells [6]. In this way, we have cloned a 2364bp cDNA encoding a 413-residue protein that is 97% identical to the human SMS1.

We found that expression of *SMS1* in yeast not only suppresses the effects of *RGS1* overexpression, but also blocks the deleterious effects of a number of other stresses including the heterologous expression of mouse Bax, treatment with hydrogen peroxide, osmotic stress and elevated temperature. The anti-apoptotic effects of SMS1 in yeast are likely due to its biochemical property converting stress-mediated accumulation of

ceramide to sphingomyelin.

Although *SMS1* cDNA sequences have been in the GenBank database prior to the identification of its function, the 5' end of the *SMS1* cDNA that we isolated in our previous study represented a novel cDNA that contains a novel 5' *UTR* sequence. This suggested that *SMS1* might be an alternatively spliced gene, giving rise to multiple transcripts. Here, we have analyzed the available *SMS1* cDNA sequences and the corresponding mouse genomic sequences in order to determine the structure of the *SMS1* gene. Our results indicate that the *SMS1* gene consists of at least 16 different exons that are alternatively spliced to generate four different transcripts and three different proteins. The regulation and possible functions of the different splice variants and proteins were also analyzed.

4. Materials and methods

4.1 Isolation of total RNA

Two-month-old Balb/c mice (Charles River Laboratories) were sacrificed by sodium pentobarbital overdose (150 mg/kg). The animals were surgically dissected, the tissues removed, washed in 1x phosphate buffered saline (PBS), frozen in liquid nitrogen and stored at -80°C. C2C12 cells were washed 3 times with ice cold PBS, collected by

centrifugation and stored at -80°C. RNA was extracted from tissues and cells using TRIzol essentially as described by the supplier (Invitrogen). The RNA was used for RLM-RACE and for RT-PCR analysis (See sections 4.2 and 4.3).

4.2 RLM-RACE

RLM-RACE was used to amplify cDNA containing the entire 5' or 3' ends of the different *SMS1* transcripts essentially as described by the manufacturer (Ambion). For 5' RACE, total RNA extracted from different tissues (skeletal muscle for *SMS1*α1, kidney for *SMS1*α2 and testes for *SMS1*ß and *SMS1*γ transcripts) was treated with calf intestinal phosphatase (CIP) for 1hr at 50°C in order to remove 5' PO_4 from degraded mRNAs and other RNAs. The RNA was then treated with tobacco acid pyrophosphatase (TAP) for 1hr at 37°C. This removes the cap, thereby allowing the ligation of a specific adapter to the 5' end of mature mRNAs. The RNA was then reverse transcribed into cDNA using M-MLV reverse transcriptase for 1hr at 42°C. The mRNA corresponding to the different SMS1 transcripts were then amplified using two separate PCR reactions. The first PCR reaction used an outer forward oligo that was specific for the RNA adapter (5'-GCTGATGGCGATGAATGAACACTG-3') and a reverse outer oligo corresponding to a specific sequence present in the different SMS1 transcripts. The PCR products were then re-amplified using an inner forward oligo that was specific for the RNA adapter (5'-CGCGGATCCGAACACTGCGTTTGCTGG CTTTGATG-3') and a reverse inner oligo corresponding to a specific sequence present in the different *SMS1* sequences. The *SMS1*

specific oligos were as follows: for *SMS1*α1, outer 5'-TGTGCTCCAAATGTACCCTGC-3' (from exon 4) and inner 5'-CCGCCGGAATTCTTTCCACGC-3' (from exon 2); for *SMS1*α2, outer 5'-ACTGTTCGTACAGGGCAGGAC-3' (from exon 9) and inner 5'-CCAAAGGCAGCCTCCTGGAAA-3' (from exon 8); for *SMS1*ß, outer 5'-GAATTCTTTCCACGCGCCGAG-3' (from exon 2) and inner 5'-ATG-CAGTCCGCCAGTCTACAG-3' (from exon 1) and for *SMS1*γ, outer 5'-ACTGTTCGTACAGGGCAGGAC-3' (from exon 9) and inner 5'-TATCAAGGCATGGCCCTGCAG-3' (from exon 5).

RLM-RACE was also used to obtain the 3' end of *SMS1*ß. In this assay, total RNA from testes was reverse transcribed into cDNA using an oligo dT containing adapter sequence. The cDNA was amplified by two separate PCR reactions. The first PCR used an outer reverse oligo that was specific for the oligo dT containing primer (5'-GCGAGCACAGAATTAATACGACT-3') and a forward outer oligo corresponding to a specific *SMS1*ß outer sequence, 5' GATGGGGACACTGAGTTTCTC 3' (from exon 10). The PCR product was re-amplified using an inner reverse oligo that was specific for the oligo dT containing primer (5'-CGCGGATCCGAATTAATACGACTCACTATAGG-3') and a forward outer oligo corresponding to a specific *SMS1*ß sequence 5'-AGCCACGGGTTTACTTGGATC-3' (from exon 10). PCR was performed at 94°C for 45sec, 60°C for 30sec and 72°C for 45sec (PCR with outer oligos) or 15 (PCR with inner oligos) for a total of 35 cycles followed by a final extension at 72°C for 30min. Aliquots of the PCR reactions were first visualized by ethidium bromide agarose gel electrophoresis and subsequently cloned into pCR2.1 (Invitrogen). Plasmid DNA

containing 5 independently isolated PCR fragments for each *SMS1* transcript was sequenced.

4.3 Reverse transcriptase polymerase chain reaction (RT-PCR)

One μg of total RNA was reverse transcribed and amplified by PCR using the ThermoScript RT-PCR system (Invitrogen). A reverse oligo 5'-CTGCCCATTGTCAGAGGAGAC-3', corresponding to a common sequence in exon 9 was used to amplify all four *SMS1* transcripts (see Figure 3.1 for location of oligos in the different transcripts). Sequence specific forward oligos as well as specific amplification conditions used for the different *SMS1* transcripts, were as follows: for *SMS1*α1 94°C for 45sec, 55°C for 30sec, 72°C for 45sec for a total of 31 cycles using the forward oligo : 5'-GGAAAGAATTCCGGCGGCTGC-3'; for *SMS1*α2, 94°C for 45sec, 55°C for 30sec, 72°C for 45sec for a total of 35 cycles using the forward oligo 5'-TGTGCTTCCCAGAGGCGGCCT-3'; for *SMS1*ß, 94°C for 45sec, 57°C for 30sec, 72°C for 45sec for a total of 32 cycles using the forward oligo 5'-TGTACCCCCGCTGTAGACTGG-3'; for *SMS1*γ, 94°C for 45sec, 57°C for 30sec, 72°C for 45sec for a total of 30 cycles using the forward oligo 5'-ACAGAGTGCAGAGGAGTCAGC-3'. As a control, ß-*ACTIN* was amplified using these conditions: 94°C for 45sec, 50°C for 1min and 72°C for 45sec for a total of 25 cycles and using the forward 5'-GTGGGCCGCCCTAGGCACCAG-3' and reverse 5'-

CTCTTTGATGTCACGCACGATTTC-3'oligos. Transcript for the second mammalian SMS encoding gene, *SMS2*, was amplified as follows: 94°C for 45sec, 55°C for 30sec, 72°C for 45sec for a total of 30 cycles using the forward oligo : 5'-CCAACGGGTTACGAAAGG-3' and reverse oligo : 5'-GCCGCTGAAGAGGAAGTC-3'. An aliquot of each PCR reaction was separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, visualized, and photographed under UV illumination.

4.4 Cell culture

The mouse skeletal muscle C2C12 cell line was used to test the effect of TNF-α on the expression of the different SMS1 transcripts. C2C12 cells were grown in DMEM supplemented with 10% Fetal Bovine Serum and 1% mixture of penicillin-streptomycin at 37°C in 5% CO_2. Cells were grown to 70% confluency and TNF-α was added to the culture medium at a concentration of 50 ng/ml. Total RNA was extracted 24h later. The RNA was used to determine the levels of the different SMS1 transcripts by RT-PCR as described in section 4.3.

4.5 Yeast strains and plasmids

Strain BY4741 (*MAT***a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) was used as the wild type

yeast cell. The yeast expression vectors to express SMS1α (pYES2-SMS1α), Bax (pGilda-Bax) as well as the corresponding empty control vectors (p425GAL1, p246GAL1 and the GFP expressing vector p425-ATG-GFP) were previously described [15,16]. The SMS1β-GFP expressing vector was constructed by amplifying the coding sequence of SMS1β by PCR using pYES2-SMS1α1 DNA as a template with the following forward 5'-CTAGTGGATCCCCCGGGCTGCAGGAATTCGGCAAGCTG GGGGTACTGAATG-3' and reverse 5'-CATGGTGGCGATGGATCCCGGGCCCGC GGTACCGTTGTATTTTAAGAGCAGCCA-3' oligonucleotide primers essentially as previously described [15]. The PCR product was ligated into the GFP vector in vivo by co-transforming the PCR product into yeast with *Hin*dIII linearlized p426GAL1-GFP as previously described [17].

4.6 Yeast growth and transformation

Synthetic minimal media consisting of Yeast Nitrogen Base (YNB) containing 2% glucose supplemented with the appropriate amino acids and bases was routinely used to grow yeast [15,16]. Glucose was replaced with 2% galactose and 2% raffinose in order to induce expression of sequences under the control of the *GAL1* promoter [14]. Plasmids were introduced into yeast using lithium acetate and the resultant transformants were selected and maintained by omitting the appropriate nutrient from the growth media. The plasmids used contained the following selectable markers: p425GAL1, *LEU2*; pGilda-Bax and p423GAL1, *HIS3*; and pYES2, *URA3*.

To determine the effects of expressing *SMS1*β on the growth of *SMS1*α and *BAX*-expressing cells, the spot growth assay was used essentially as described [14]. Briefly, freshly saturated glucose cultures of the different transformants were serially diluted (1 to 5) and 10µl aliquots of each dilution was spotted on nutrient agar plates containing either glucose or galactose and subsequently incubated at 30°C for 3 to 4 days.

4.7 Western blot analysis

Soluble yeast protein was extracted by heating NaOH treated yeast cells in SDS-PAGE loading buffer for 3 min at 95°C [18]. An equal amount of protein of the different samples were separated by SDS-PAGE, transferred to membrane and sequentially challenged with monoclonal anti-GFP antibody (Santa-Cruz Biotech) and with HRP-conjugated donkey anti-goat IgG secondary antibody. Signals were subsequently developed using chemiluminescent luminol reagent (ECL+) and exposure to X-ray film.

5. Results and Discussion

5.1 Organization and alternative splicing of the SMS1 gene

In addition to the cDNA identified in our previous study (GenBank accession No. AY509044), three other mouse *SMS1* encoding cDNAs that differ in their 5' and 3' non-coding sequences were found in the GenBank database (accession No. BC019443, AK082974 and AK076554). These differences suggested that the cDNAs represent mRNAs that originated from alternative splicing of a single gene.

In order to characterize the observed differences as well as the structure of the *SMS1* gene, we used BLAST to compare the nucleotide sequences of the four *SMS1* cDNAs with the mouse genomic database. All *SMS1* cDNA sequences were located within 16 different exons that were spread over a 264kb portion of mouse chromosome 19 (GenBank accession No. NT039687) (Figure 3.1A). The four cDNAs show significant differences in the combinations of exons used (Figure 3.1B). Analysis of the predicted open reading frames of the *SMS1* cDNAs suggested that there also exist three different SMS1 proteins. A nomenclature was adopted in order to differentiate between the different SMS1 transcripts and proteins (Figure 3.1B, Figure 3.2). The alternatively spliced *SMS1*α mRNAs, *SMS1*α1 and *SMS1*α2, refer to the two different transcripts that code for the previously characterized 413-residue SMS1α protein (Figure 3.1B, Figure 3.2). *SMS1*ß and *SMS1*γ refer to mRNAs that encode C-terminally truncated forms of the

SMS1α protein. SMS1ß consists of the N-terminal 208 residues of SMS1α. SMS1γ is identical to SMS1ß except that there is an addition of the tripeptide LSP to its C-terminus. Both SMS1ß and SMS1γ contain the N-terminal Sterile Alpha Motif (SAM) domain as well as the first two of the six transmembrane domains (TMDs) found in SMS1α. A schematic diagram is shown in order to illustrate the structural differences between the different SMS1 proteins (Figure 3.2).

Of the 16 *SMS1* exons, numbers 1 through 8 contain all the non-coding 5' *UTR* sequences. Alternative splicing of these exons gives rise to different and unique 5' *UTR*s for the 4 *SMS1* mRNAs (Figure 3.1B). Exon 9, the only exon that is common and present in all 4 mRNAs, contains the translational start site, the SAM motif as well as the first two putative TMDs of SMS1. Exon 9 is spliced onto exons 13 through 16 to make the 3' ends of both *SMS1*α1 and *SMS1*α2 transcripts. The 5' *UTR* of *SMS1*ß consists of exons 1, 3, 4, 6 and 7 while exon 9 is spliced onto exon 10 to make up the coding portion as well as the 3' *UTR* of the transcript (Figure 3.1B). The 5' *UTR* of *SMS1*γ consists of exons 5, 6 and 7 while exon 9 is spliced onto exon 11 to make up the coding portion and 3' *UTR* of the transcript. Exons 12 through 16 make up the 3' *UTR* of the *SMS1*γ transcript (Figure 3.1B).

5.2 Mapping the 5' and 3' ends of the SMS1 transcripts

Although the 5' and 3' ends of most *SMS1* exons were readily identified by

comparing the sequences of the cDNAs and genomic DNA, the 5' ends of the different transcripts are not available by using such an analysis. This is because the 5' ends of the different *SMS1* transcripts are encoded by unique exons. For example, exon 2 contains the 5' end of *SMS1*α1, exon 8 contains the 5' end of *SMS1*α2, exon 1 contains the 5' end of *SMS1*ß and exon 5 contains the 5' end of *SMS1*γ (Figure 3.1B). We therefore used a modified 5'RACE, RLM-RACE, to identify the 5' ends the *SMS1* transcripts [19,20]. This technique involves the ligation of a specific linker onto the 5' ends of mature transcripts, which then permits the selective amplification of capped mRNA. Using a 2-step amplification process with different combinations of oligos corresponding to outer and inner segments of the ligated linker as wells as outer and inner oligos corresponding to the desired transcript, we amplified PCR product corresponding to the 5' ends of all four alternatively spliced *SMS1* mRNAs.

An aliquot of each PCR reaction was separated by agarose gel electrophoresis (Figure 3.3A). Single bands of 300, 300, 400 and 100bp corresponding to *SMS1*α1, *SMS1*α2, *SMS1*ß and *SMS1*γ transcripts were respectively amplified. The PCR products were subcloned and the nucleotide sequence determined from five different clones for each transcript. The deduced nucleotide sequences were compared to the sequences of the 5' ends of the *SMS1* cDNAs as well as to the nucleotide sequences of the corresponding region of the mouse genomic DNA (Figure 3.3B). The available sequences of the 5' ends of the different transcripts were extended by the following: 51nt for *SMS1*α2 (GenBank accession No. BC019443), 194nt for *SMS1*ß (GenBank accession No. AK082974) and 7nt for *SMS1*γ (GenBank accession No. AK076554). The nucleotide sequence of the RACE PCR products suggested that the 5' end of the *SMS1*α1 cDNA (GenBank

accession No. AY509044) corresponds to the 5' end of the transcript.

Exon 16 contains the 3' end of 3 of the 4 *SMS1* transcripts including *SMS1*α1, *SMS1*α2 and *SMS1*γ (Figure 3.1B). The nucleotide sequence of the *SMS1*α1 cDNA includes a polyA tail that is not present in the corresponding genomic sequence. In addition, a consensus polyadenylation sequence (AATAAA) is located 19nts upstream of the 3' polyA tract [21]. This indicates that we have identified the 3' end of the transcripts containing exon 16 at their 3' ends.

The 3' end of *SMS1*ß is unique since it consists of exon 10 (Figure 3.1B). There is no polyA tail present at the 3' end of the *SMS1*ß cDNA sequence. We therefore used RLM-RACE to amplify, clone and determine the 3' end of the *SMS1*ß transcript (Figure 3.3A). Sequence analysis of the cloned 3' RACE *SMS1*ß PCR product allowed us to extend the 3' end of the cDNA sequence by 21nt (Figure 3.3B). Although this sequence has a polyA sequence at its 3' end, this sequence is present in the genomic DNA (Figure 3.3B). In addition there is no consensus polyadenylation sequence within the 500nt either 5' or 3' of the end of the *SMS1*ß sequence. Although the *SMS1*ß 3' RLM-RACE PCR product clearly consists of a single band when visualized on an ethidium bromide stained agarose gel (Figure 3.3A), this band apparently does not represent the 3' end of *SMS1*ß. We cannot explain the absence of a polyA tail at the 3' end of the amplified *SMS1*ß transcript.

The sequences of the intron/exon boundaries as well as the sizes of the different exons were determined by comparing the sequences of the *SMS1* transcripts and the corresponding genomic DNA (Table 3.1). With the exception of the boundary between exon 9 and 10, all the exon/intron boundaries follow the GT/AG rule for splice sites. The

exception reflects the fact that there is no intron between exons 9 and 10. Exon 10 is therefore both an exon as well as an intron. The splice sites of these coding introns may differ from the typical introns [22].

5.3 Tissue distribution of the SMS1 transcripts

In order to confirm that the cDNAs represent expressed transcripts, semi-quantitative RT-PCR analysis was performed using total RNA isolated from a panel of mouse tissues. PCR products corresponding to the predicted size were detected for all four cDNAs, suggesting that they represent expressed transcripts (Figure 3.4). The *SMS1*α1 and *SMS1*ß transcripts are widely distributed, the *SMS1*α2 transcript show a more narrow distribution while the *SMS1*γ transcript is detected only in testes and heart. Taken together, these results suggest that the regulation and functions of the different SMS1 transcripts and proteins are likely to be tissue specific. Mammalian cells also contain a second SMS gene called *SMS2* [3]. Although SMS2 has SMS activity, it lacks the N-terminal SAM domain present in SMS1. We also examined the tissue distribution of *SMS2* transcript using RT-PCR. The *SMS2* transcript is widely distributed indicating that many cells are likely to express both *SMS1* and *SMS2* (Figure 3.4).

5.4 Regulation of SMS1 gene expression in response to TNF-α.

SMS catalyzes the production of sphingomyelin and diacylglycerol (DAG) using ceramide and phosphatidylcholine as substrates [7,8]. Activation of SMS prevents apoptosis by a mechanism that likely involves a decrease in the levels of the pro-apoptotic ceramide [8,13]. Regulation of SMS activity has been reported to occur in response to both mitogenic and apoptotic stimuli [23-25]. Mammalian cells contain two SMS genes namely *SMS1* and *SMS2* [3,7].

Since the *SMS* genes have only recently been cloned [3,4], the regulation of either enzyme has not been fully addressed. As a first step towards examining the regulation of *SMS1*, we determined the levels of *SMS1* transcripts in cultured C2C12 cells stimulated with the pro-apoptotic cytokine TNF-α. Total RNA was extracted from untreated C2C12 cells as well as C2C12 cells treated with TNF-α for 24 hours. The RNA corresponding to the different *SMS1* transcripts were amplified by RT-PCR using transcript specific oligos. Three of the four *SMS1* transcripts, α1, α2 and β, were detected in control C2C12 cells (Figure 3.5A). A modest increase in all 3 transcripts is detected in cells treated with TNF-α. *SMS1*γ was not detected in either control or TNF-α treated cells. As a control, we also amplified the transcript corresponding to β-*ACTIN*, the levels of which were found to remain constant in control as well as TNF-α treated cells. As a control, we also show that TNF-α induced the cleavage of PARP and caspase-3, indicating that TNF-α is capable of initiating apoptosis in these cells (Figure 3.5B). These results suggest that the *SMS1* gene may be transcriptionally regulated by pro-apoptotic stimuli like TNF-α. Given that SMS1

is likely to be anti-apoptotic, an up-regulation of the levels of *SMS1* transcripts may represent a response to stress that attempts to counteract the stress and in order to try avoiding cell death. Such an up-regulation in response to stress has been observed for a number of other anti-apoptotic genes in response to apoptotic stimuli [26,27]. The regulation of *SMS1* gene in other cells and in other conditions remains to be determined.

A previous study has demonstrated that an increase in the nuclear levels of ceramide is responsible for Fas-induced apoptosis in Jurkat T-cells [25]. A decrease in nuclear, but not microsomal, SMS activity correlated with the stress mediated increase in ceramide. The loss of SMS activity was rapid, since over half the SMS activity disappeared within 6h of Fas-induced apoptosis. This indicates that post-translational mechanisms also serve to regulate the levels of SMS. Although the molecular mechanisms responsible is not known, it is interesting to note that preventing caspase 3 activation served to inhibit the loss of SMS activity. It remains to be determined the importance of this process in other cell types as well as the relative contribution of *SMS1* and *SMS2* to the process.

5.5 Analysis of SMS1β protein in yeast

The 413 residue SMS1α protein has been shown to possess sphingomyelin synthase activity when overexpressed in either mammalian or yeast cells [3,4]. The function of the truncated SMS1 proteins encoded by the *SMS1*β and *SMS1*γ transcripts has not been investigated. The SMS1β and SMS1γ proteins contain the first two of the six putative

TMDs found in SMS1α (Figure 3.2) and are therefore unlikely to have sphingomyelin synthase activity [3].

Like SMS1α, these truncated proteins, SMS1β and SMS1γ, nevertheless contain the N-terminal SAM motif. The ca. 70 amino acid SAM domain has been shown to interact with SH2 or other SAM domains [28]. This motif is present in a large number of different proteins, many of which are involved in signal transduction. For example, the yeast SAM containing Ste50 protein is involved in modulating a number of signaling responses including the ability to respond to mating pheromone. The ability of Ste50p to interact with the Ste11p kinase through their common SAM domain is necessary for its function [29]. It is known that proteins containing SAM domains, such as SMS1, are capable of forming both homodimers as well as heterodimers [28]. Thus it is possible that SMS1β exerts its function by interacting with SMS1α. Such an interaction may lead to an increase or a decrease in SMS1α function.

We have previously demonstrated that SMS1α inhibits apoptosis in response to a number of stimuli including the expression of murine Bax in yeast [6]. We therefore used the spot growth assay to examine the possibility that SMS1β may decrease or enhance the ability of SMS1α to prevent Bax-mediated cell death in yeast. Yeast cells were transformed with empty plasmids or with different combinations of *BAX*, *SMS1α* and *SMS1β* expressing plasmids. The transformants were grown to saturation in glucose containing yeast media, serially diluted (5 fold), and 10μl aliquots were spotted onto selective nutrient agar plates containing either glucose to repress the expression or galactose to induce the expression of *BAX*, *SMS1α* and/or *SMS1β*. The plates were then incubated at 30°C and the cells allowed to grow for 3 to 4 days. No difference was

observed in the growth of all the transformants on glucose-containing media (Figure 3.6A, left panel). Similarly, no differences were observed between the growth of control, *SMS1*α or *SMS1*β-expressing cells on galactose media (Figure 3.6A, right panel). In contrast, the growth of cells expressing Bax was inhibited on galactose media. As previously demonstrated [6], co-expression of *SMS1*α largely abolished the growth inhibitory effects of Bax (Figure 3.6A, right panel). In contrast, cells co-expressing *SMS1*β with *BAX* showed the same growth inhibitory effects as cells expressing Bax alone. Yeast cells co-expressing *SMS1*β along with *BAX* and *SMS1*α showed the same growth as cells co-expressing Bax and SMS1α. These results indicate that SMS1β is unable to prevent the growth inhibitory effects of Bax and nor does it interfere or enhance the effects of SMS1α on Bax.

As a control, we performed western blot analysis on cells harboring the *SMS1*β construct. The *SMS1*β used in this study was expressed as GFP fusion. Since no antibodies are available for SMS1, this allowed us to follow the expression of *SMS1*β using GFP antibodies. In addition, the *SMS1*β-*GFP* fusion is expressed under the control of the *GAL1* promoter since it allows us to induce its expression by the addition of galactose. Using a GFP specific antibody, we detected a 53kDa band corresponding to the appropriate size for the SMS1β-GFP protein only in galactose grown cells containing the plasmid encoding the *SMS1*β-*GFP* gene fusion (Figure 3.6B). This suggests that the inability of *SMS1*β to affect function of SMS1α is not due to its inability to be expressed in yeast. Although the GFP tag may interfere with SMS1β function, numerous studies have shown that such a tag does not commonly interfere with protein function [16,30].

5.6. Conclusions

The *SMS1* gene was found to consist of 16 different exons that are alternatively spliced to yield 4 different transcripts and 3 different proteins. Given that SMS is an important enzyme in sphingolipid metabolism which plays a central role in regulating the levels of the pro-apoptotic ceramide and the mitogenic DAG [7], the complexity of the *SMS1* gene suggest that a number of different processes are involved in regulating the expression of this gene and protein.

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7. Figure Legends, Figures and Table

Figure 3.1 Schematic representation of the genomic organization of the mouse *SMS1* gene.

The sequence of the different mouse *SMS1* cDNAs were used to identify the *SMS1* gene in the mouse genomic DNA database. **A.** The *SMS1* gene is located on a 265kb fragment of DNA on chromosome 19. Exons are shown as numbered boxes. The sizes of the intervening (introns) are represented by lines and their lengths are shown in base pairs. There is no intron between exons 9 and 10. **B.** The exon composition of the different *SMS1* cDNAs is shown. *SMS1α* refers to the cDNA that we previously cloned while the other cDNAs were named *SMS1α2*, *SMS1ß* and *SMS1γ* to reflect the different exons used and the different proteins. The location of the putative "ATG" that is used as the translational initiation site for all 3 forms of the SMS1 protein is shown in exon 9. Shaded areas within the different exons represent regions that are used as coding sequence. Arrows denote the location of primers used to amplify the different mRNAs by RT-PCR (see Figure 3.4).

Figure 3.2 Schematic representation of the SMS1 proteins.

Alternative splicing of the *SMS1* gene gives rise to 4 different transcripts as well as 3 different proteins that we have named SMS1α, SMS1ß and SMS1γ. SMS1α contains 413 residues and is predicted to be an integral membrane protein. The topology as well as the

N-terminal Sterile Alpha Motif (SAM) of SMS1α is shown. SMS1ß and SMS1γ refer to C-terminally truncated forms of the SMS1α protein. SMS1ß consists of the N-terminal 208 residues of SMS1α. SMS1γ is identical to SMS1ß with the addition of the tripeptide LSP to its C-terminus. The N-terminal SAM domain as well as the two predicted N-terminal TMDs of SMS1ß and SMS1γ are shown.

Figure 3.3 RLM-RACE amplification and analysis of the 5' and 3' ends of the *SMS1* transcripts.

For 5' RACE, an RNA adapter was ligated onto the 5' end of total RNA. The RNA was then made into cDNA using reverse transcriptase. For 3' end RACE, the RNA was made into cDNA using reverse transcriptase and an oligo dT containing adapter sequence. The RNA was then amplified by 2 separate PCR reactions using both outer and inner oligos corresponding to the RNA adapter or the oligo dT containing adapter sequence and to the different *SMS1* transcripts. **A.** An aliquot of the final PCR products of 5'RACE (*SMS1*α1, *SMS1*α2, *SMS1*ß and *SMS1*γ) and 3' RACE (*SMS1*ß) reactions were separated by agarose gel electrophoresis and a photograph of the ethidium bromide stained gel is shown. The sizes of the molecular weight markers used are shown on the left of the gel. **B.** An aliquot of the PCR products were ligated into pCR2.1 and the nucleotide sequence of five plasmids containing the different insert were determined. The nucleotide sequences were compared to the corresponding GenBank cDNA sequences and the appropriate mouse genomic sequence. The complete sequence of the exons containing the 5' ends and the 3' end of the *SMS1* transcripts analyzed are shown. The underlined sequence corresponds to

new sequences determined by RLM-RACE and corresponds to an extension of the sequences present on the cDNA sequences.

Figure 3.4 Tissue distributions of the *SMS1* transcripts.

Total RNA was isolated from a variety of mouse tissues, reverse transcribed and the different *SMS1* transcripts were amplified using the ThermoScript RT-PCR system (Invitrogen). A reverse oligo corresponding to a sequence in exon 9 that is present in all four *SMS1* transcripts as well as transcript specific oligos were used to amplify all four *SMS1* transcripts (see Figure 3.1 for the locations of the oligos). As a control, ß-*ACTIN* was also amplified. An aliquot of each PCR reaction was separated by agarose gel electrophoresis, stained with ethidium bromide and a composite of the resultant photographs are shown. The size in base pairs (bp), of each PCR product corresponding to the predicted size of the different *SMS1* transcripts is shown on the right. Similar results were obtained in 2 separate experiments.

Figure 3.5 Analysis of the effects of TNF-α on the expression of *SMS1* transcripts.

A. Total RNA was extracted from untreated (-) as well as TNF-α treated C2C12 cells. The mRNAs corresponding to the different *SMS1* transcripts were amplified by RT-PCR using transcript specific primers (see legend to Figure 3.3). An aliquot of each PCR reaction was separated by agarose gel electrophoresis and a composite of the ethidium bromide stained gels is shown. The levels of β-*ACTIN* were also determined and served as a loading control. **B.** Soluble protein was extracted from untreated (-) as well as TNF-α

treated C2C12 cells. The extracts were analyzed by western blot using a monoclonal anti-PARP antibody (top panel) which recognize both the full length (inactive) and cleaved PARP (89kDa) and a monoclonal caspase 3 antibody that recognizes both the pro-caspase (35kDa) as well as the cleaved form of caspase 3 (17kDa).

Figure 3.6 Functional expression and analysis of SMS1β in yeast.

A. Yeast transformants that contain only empty p423GAL1, p425GAL1ATG-GFP and pYES2 vectors (CONTROL) were compared to cells harboring either plasmid p423GAL1, p425GAL1ATG-GFP and pYES2-SMS1 (SMS1α); p423GAL1, pYES2 and p425GAL1-SMS1β-GFP (SMS1β); pGildaBax, pYES2 and p425GAL1ATG-GFP (Bax); pYES2-SMS1, pGildaBax and p425GAL1ATG-GFP (SMS1α/Bax); p425GAL1-SMS1β-GFP, pGildaBax and pYES2 (SMS1β/Bax); and p425GAL1-SMS1β-GFP, pYES2-SMS1 and pGildaBax (SMS1α/SMS1β/Bax). Yeast cells were grown to saturation in YNB glucose containing media, serially diluted and 10μl of each dilution were spotted onto selective nutrient agar plates containing either glucose (GLU) or galactose (GAL) to induce *BAX*, *SMS1α* or *SMS1β-GFP* expression. Photographs of the resultant plates are shown after 3-4 days of incubation at 30°C. **B.** Levels of SMS1β-GFP protein in different transformants was determined in glucose (GLU) or galactose (GAL) grown cells by western blot analysis using GFP specific antibody. SMS1β-GFP was detected only in galactose grown cells harboring p425GAL1-SMS1β-GFP. Arrow depicts the location of the SMS1β-GFP protein.

Figure 3.1 The genomic organization of the mouse *SMS1* gene.

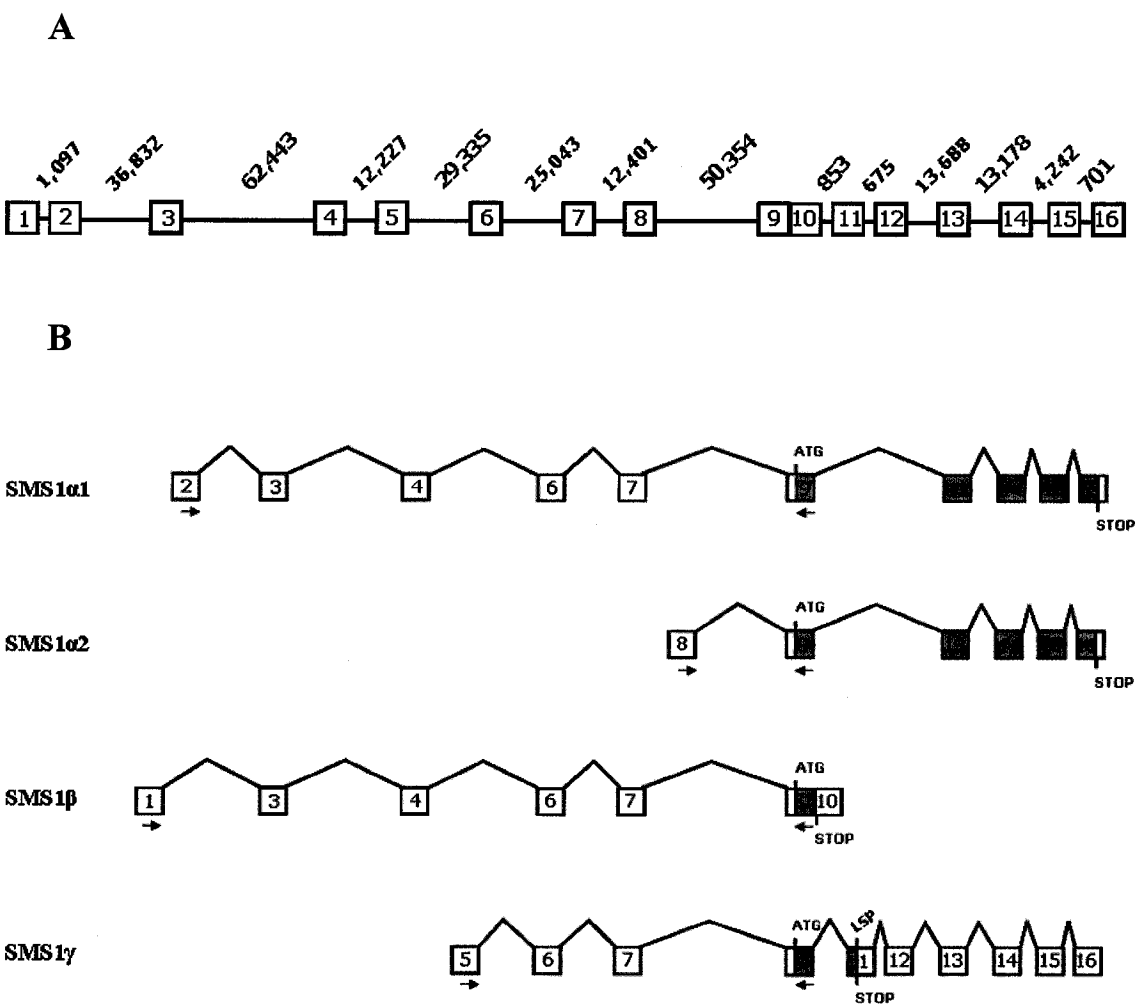
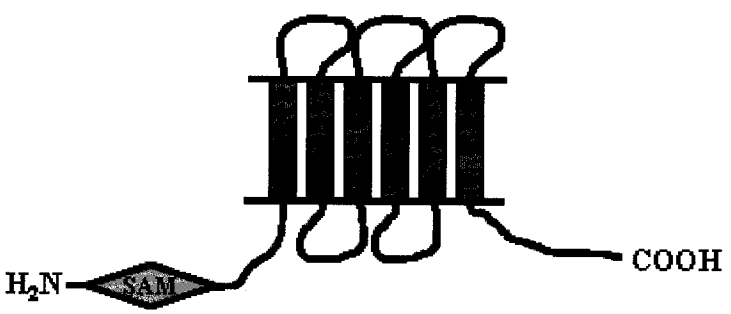
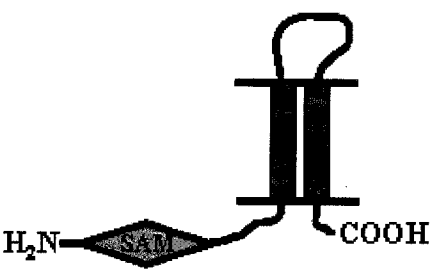


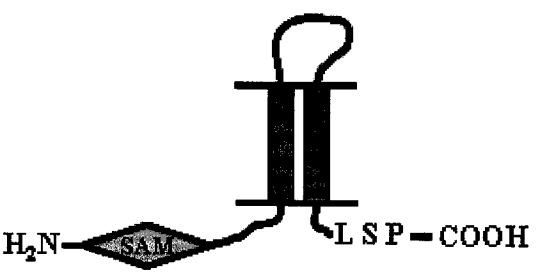
Figure 3.2 Schematic representation of the SMS1 proteins.



SMS1α



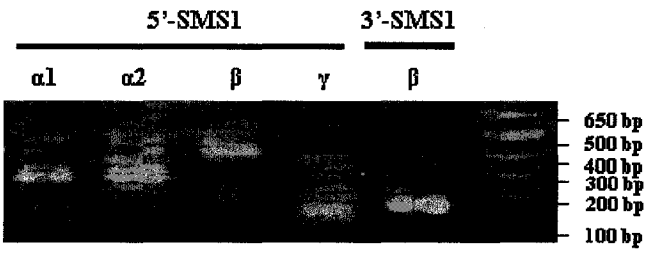
SMS1β



SMS1γ

Figure 3.3 RLM-RACE amplification and analysis of the 5' and 3' ends of the *SMS1* transcripts.

A



B

Exon 8: 5' end of SMS1α2

CTACATTCCAAAAACCCTGTGCAGCCTGAGGCGCGCGGGCCGTGCTCCTGT*AGTCCCTTGAGCTCCG
TGTCCTCTGCGTCTGGCTGTGCTTCCCAGAGGCGGCCTGTGGGGACCGCTGGCCACTCCCAACCGCTG
GCCAGCTGCACGCAGAGACCCGCGTAAACTTGTCAGGAACTTTCCAGGAGGCTGCCTTTGGGAGGAG
AG

Exon 1: 5' end of SMS1β

CCAGAGATTCCAGGACCCCAGGTGACACCATCACCATCCAAGCTAACCAGCAGCAGGAGAAAAGCAT
GGAGGAGAGCGACGGCTGTCCAAACATCTTTCGCCACCTTCATCCTCCGTCGCCGGGAATGGGTCAGA
ACAGGGAAGCAGCAGGAGGAGCGATGCCAGCGCCTTAGGAACGGGAACTCCTCTTCCCA*ACTTCCG
CCCCTGGGGAAAAGCAGAAGCCATCAGCTCCAAGAGAGCGGCTCTCCAATTCTTAGCCGTTAGGTTCT
GGCTCTGGAGACAGCCGGTCCTCCCGGTGTCACCGGCGTCGCAGTACCCTCGCTGTACCCCCGCTGTA
GACTGGCGGACTGCATGCAG

Exon 5: 5' end of SMS1γ

CAGGGAA*GGGCAGCTTGAGGTCACCACAGAGTGCAGAGGAGTCAGCGTCCTGCAGGGCCATGCCTT
GATATGTCG

Exon 10: 3' end of SMS1β

GTAAGTAGAGTAAACACTCTTGAGATCCAGCCATGAAGGGGTTTGCACACAGGGATCCAGTTATTGG
AAATGAAATTTACCAAGTGTTGAAATCAAAGCTTAGGAATTCCCTTTCCTTTCCATTCTGAAGCCACA
CACCCATGGAACTAATTGAAGCTCCTACAGCTTTGAGAGGATGATGTATTTGTTGCCTGTTCGCTACA
AGATTGCTCCAGAATTAAGACCATGAGCAATGCTAGTCTATTTTCAAGCAGTAGGAAAGAAAACACT
TAGACATCTTCAGAGAGCTGAGGCTGTTGAGTAACAGAGGGGGAAACACCTACCTAGTCTTGAATGG
TGTTTACTCAGGTGAATTGTTGCCAACCTCCCACCCCTGTCAGACCTAGAACCTCATCACTAAGTATTC
ACAGTTTTTGACATGTTTTAGACAGATATTCTACAATGACTTTGCTTGGGAAAAATTTGATTATCTCAT
TTAATTTAAGCAGGAAGCATGGTGCAGAATCTATTTGATTCCATCTTACAGGATGTGTGGATAAGTAC
CTGTATTCACTTTTAGTTGGTTCCACTGTGATTTTCACGTCCCAAGCTCTAAACACAACGGGGACCGCC
CAAAGGGAGCTGTTTACTTAGGAACACCTCGTCCCTTCCTTTAATTAACTGCGCTTGTTCATGCTGTCT
GGTTGTCTTCCGAGTGGACCACCTTGTGGCATTCCTGGATGGTGTTGGGAATTACAGTTTCTCCCTACC
CACTGGATGTAGGAGCATCAGTAGGAGGTACTAGCCACGGCCCAAGCTTGCTGCTCTTGTGCCTTGGA
CAGTGTCACCAAATCCACAGAGTGAAAAAGGCTATCCCTGGGACTGAGGCACTCATCCCTGGAACTG
CCACTCTCTGTGTCACTCTGTGTGTCTGCTACTGATGATGGGGACACTGAGTTTCTCTGTAGTAGACTG
AGTTGCCTGAGCCCAAGTATGGCTGGCTATTAAAAGGAGTCATATTCCATCAAGAAAACAAAATGCA
GGTGGAGTGTTCCTGTTGAACTTTTCTGAGTGGTTTCCTGGTGTTGGATAAAGTCTCTCAGCTTATGAA
GTCATGTGTCTTCAAGCAGAAGGAGTAGGCCCCTGCTGGCTTTGCCAGGGGACCCTCGTCCCTGTGAG
AAGTCACTGACATAGCAGCCACGGGTTTACTTGGATCTAGCTTGACAATTTTATAAGTGAAGTAGAAG

TTTGAATAGAATTGATGGGCTATGCTACCGTGGAGGTGTTGG*AGAAAAAAAAAGGAAAAAAAAA

Figure 3.4 Tissue distributions of the *SMS1* transcripts.

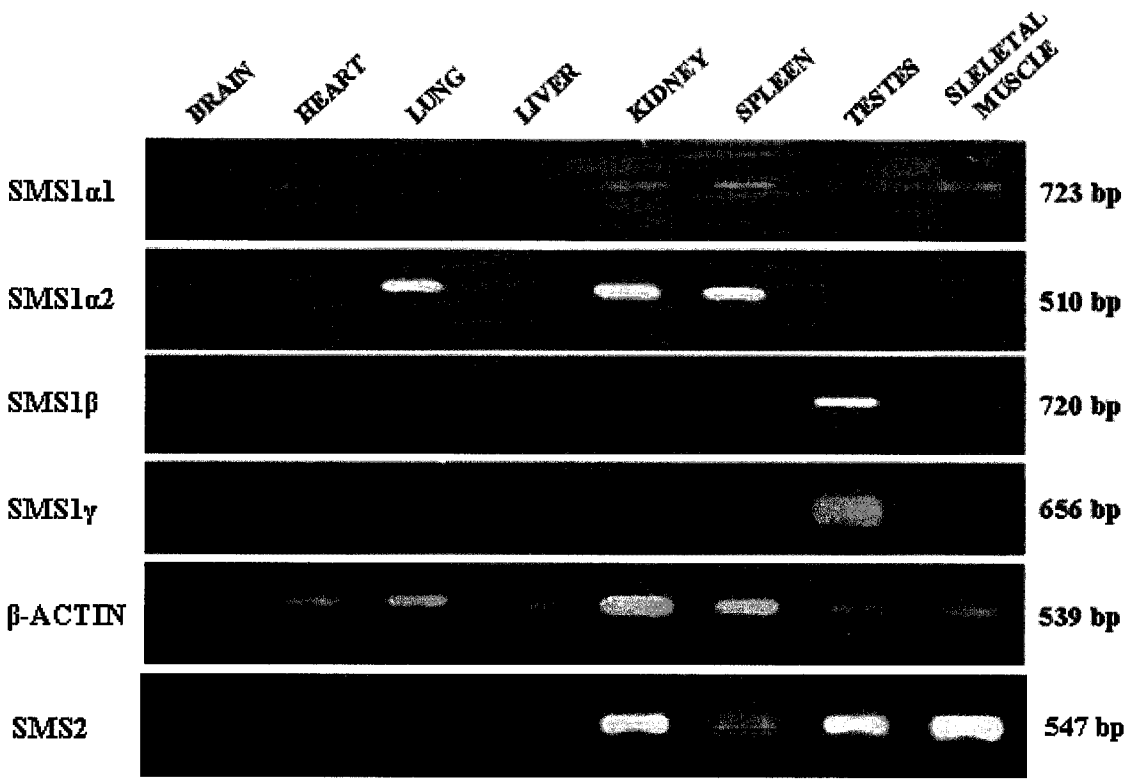


Figure 3.5 Analysis of the effects of TNF-α on the expression of *SMS1* transcripts.

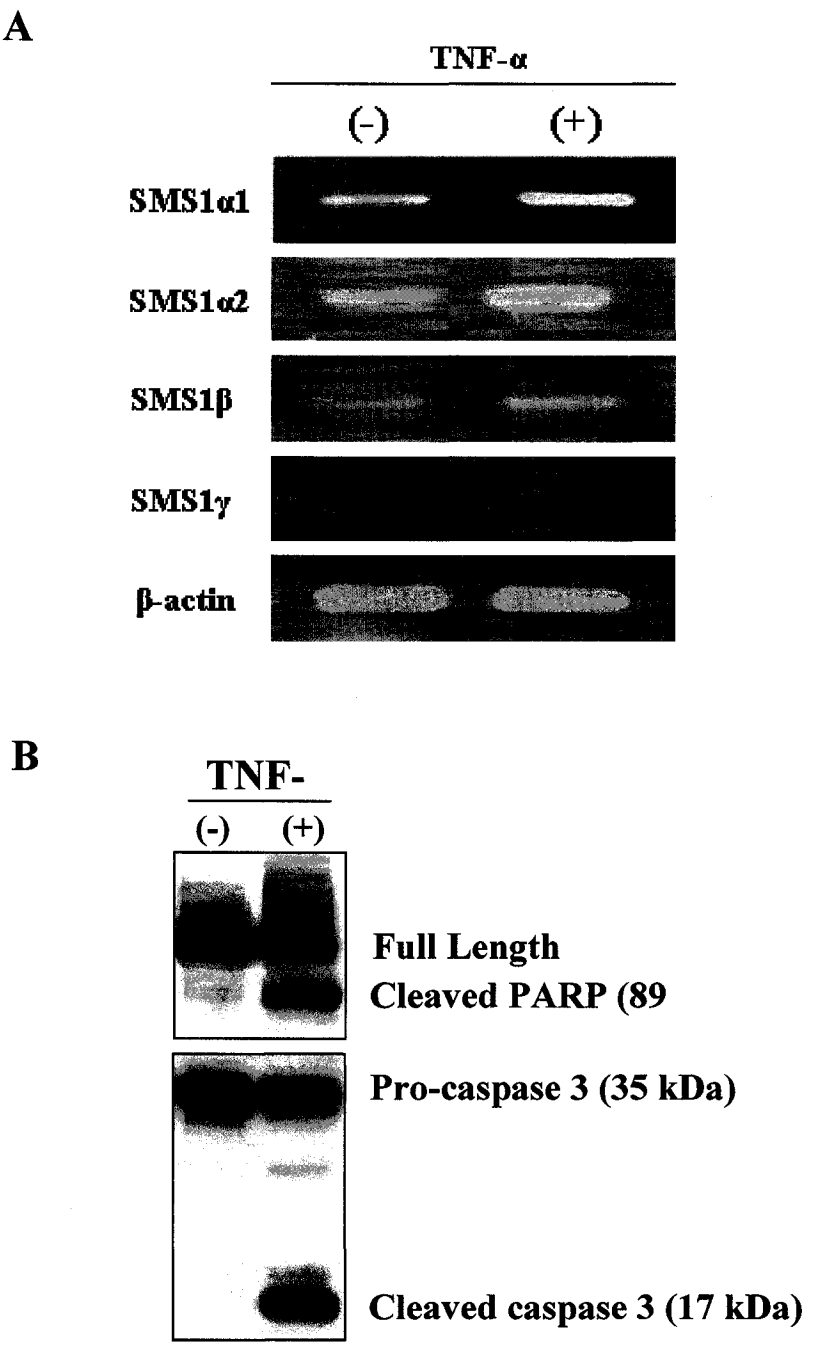


Figure 3.6 Functional expression and analysis of SMS1β in yeast.

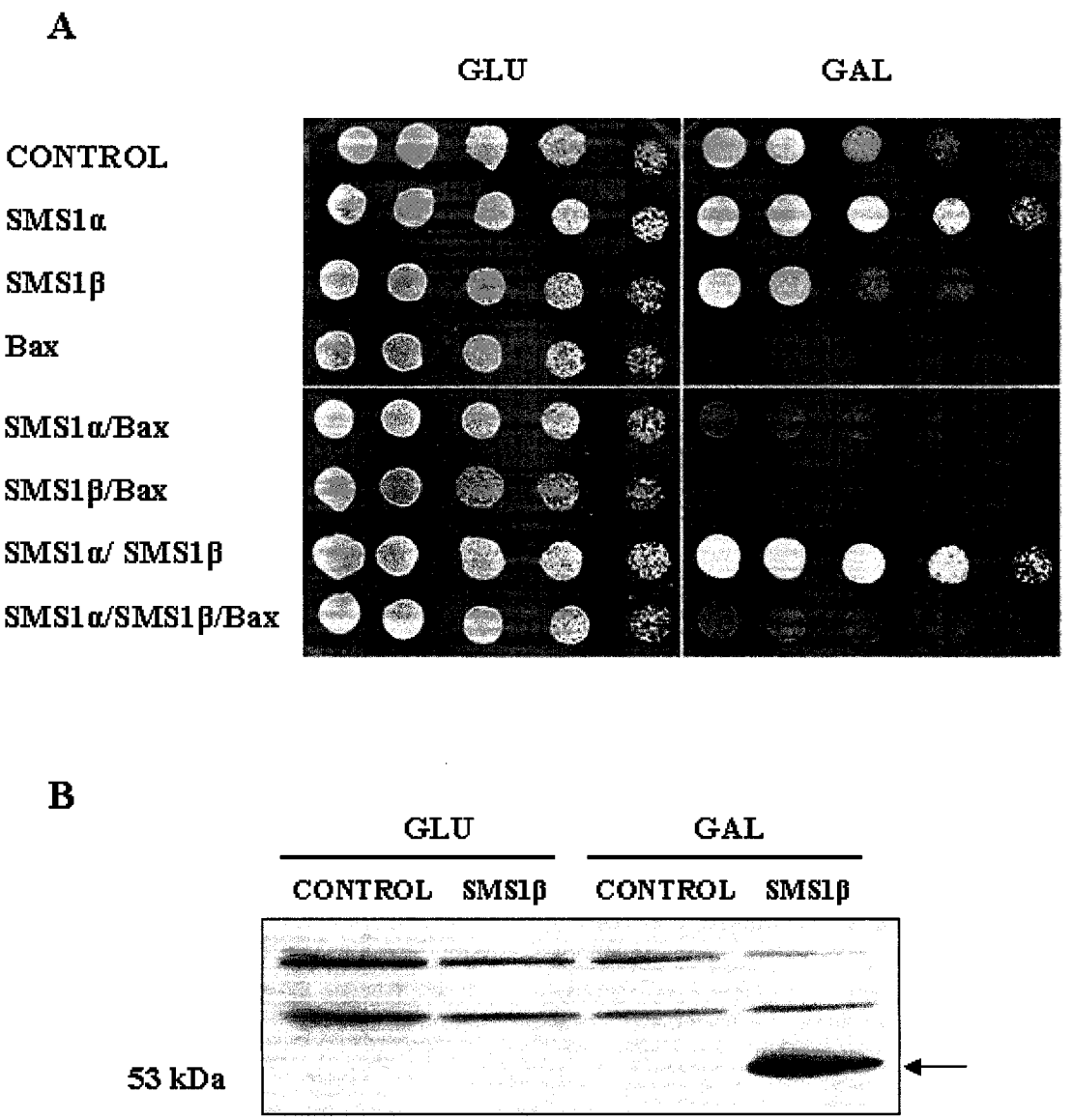


Table 3.1

Sequences of the exon-intron junctions of the alternatively spliced mouse *SMS1* gene.

exon[a]	exon size (bp)	splice acceptor site (intron-EXON)[b]	donor acceptor site (EXON-intron)
1	357	5'end=CCAGAGATTC	CTGCATGCAG-gtgagggcgg
2	244	5'end=GAGGTTCCAA	TCGCGTGCAA-gtgagtcgcg
3	79	gtgcttgcag-GGTGAAGGCA	GACTCCGAAG-gtgagtagag
4	91	gttattctag-GGAAAGCTTC	GACAGCACAG-gtaagtgaaa
5	75	5'end=CAGGGAAGGG	TGATATGTCG-gtatgggttt
6	43	tttctcctag-ACCGGAAGAA	CATGATTGAG-gtaaatggac
7	82	ccggtaacag-GTAATGGAGA	AAAATAATCC-gtaagtaaaa
8	204	5'end=CTACATTCCA	GGGAGGAGAG-gtacgtgtgg
9	840	gctcccacag-AAGGAAGAAT	TAAAATACAA-gtaagtagag
10	1290	taaaatacaa-GTAAGTAGAG	AAAAAAAAAA=3'end
11	57	ttggaagcag-ATTGTCGCCT	CCCAGTACTG-gtatgttgca
12	78	ccccatttag-AATTGGACTT	TGCAAACCAG-gtacccctg
13	118	ctttttctag-GTCTATTATT	TTCTCCGAAG-gtaaactact
14	154	tttctggtag-CTCTTTGGAG	ATCAAAGAGT-gtaagtctta
15	167	ctccttctag-ATTCTCCTCG	CAATCAGCAA-gtgagtcgcc
16	544	gctttcctag-GTGCTTAAGG	CTTGGGAAAA=3'end(A_n)

The intron/exon boundaries were determined by comparing the sequences of all the *SMS1* cDNAs (see Figure 3.1) with the mouse *SMS1* containing genomic DNA sequence found on chromosome 19 (GenBank accession No. NT039687). Introns are shown in lower case and exons in upper case letters.

[a] The 5' end of exons 1, 2, 5 and 8 contain the respective 5' ends of the transcript for *SMS1*β, *SMS1*α1, *SMS1*γ and *SMS1*α2. Exon 10 contains the 3' end of *SMS1*β while exon 16 contains the 3'end of *SMS1*α1, *SMS1*α2 and *SMS1*γ. RLM-RACE PCR was used to determine the location of the 5' ends of all 4 *SMS1* transcripts as well as the 3' end of *SMS1*β. The 3' end of exon 16 was identified by the presence of a poly A tail in the *SMS1*α1 cDNA used in this study.

[b] There is no intronic sequence between exons 9 and 10.

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Chapter 4

Evidence for the dimerization of human Regulator of G-protein Signaling 5 (RGS5).

* This chapter is correlated with the manuscript: **Zhao Yang, Sabrina Gaudio, Wei Song, Matthew Greenwood, Gaël Jean-Baptiste and Michael T. Greenwood.** Evidence for the dimerization of human Regulator of G-protein Signaling 5 (RGS5). *Cell. Physiol. Biochem.* 2007, 20(5):303-310.

1. Preface

GPCR dimerization has been confirmed to happen during the GPCR signal transduction process [1]. Either homo- or hetero-dimers are the requested functional format of GPCRs on the cell surface [1,2]. RGSs have also been know to bind with Gα subunit, GPCR or scaffolding proteins, such as spinophilin, and form large protein complexes that serve to modulate GPCR signaling [3]. However, when our lab developed human RGS5 anti-sera [4], the western blot analysis result suggested the possibility of RGS5 dimerization. I, therefore, performed a series of experiments to further investigate this hypothesis.

2. Abstract

RGS5 is a R4 type RGS that regulates GPCR signaling. Using western blot, we detected RGS5 as a specific 23 kDa protein in cells overexpressing RGS5. A 42 kDa band representing a possible RGS5 dimer was also detected. Given that GPCRs and their associated proteins form complexes involving multiple protein-protein interactions, we investigated the possibility that the 42 kDa band represents an RGS5-RGS5 dimer. RGS5 dimerization was confirmed by the analysis of a GFP tagged RGS5 fusion in yeast and with two-hybrid assays. Analysis of RGS5 in HEK293A cells suggests that the dimer may serve a regulatory function since it is longer lived than the monomer.

Key words: RGS, RGS-GFP, RGS dimer, Yeast, Heterologous expression.

3. Introduction

Regulators of G-protein Signaling (RGS) refers to a conserved 120-125 residue motif that was first identified for its ability to negatively regulate G-Protein Coupled Receptor (GPCR) signaling [5-7]. There are now well over 25 RGS containing mammalian proteins that are categorized into several distinct sub-families [8-10]. Many of these RGSs are large proteins that also harbour other functional motifs such as PDZ, PH and DEP domains [11]. This implies that these RGSs interact with multiple protein partners. On the other hand, members of the R4 subfamily of RGSs are small proteins (ca. 200 residues) that contain little more than an RGS motif [8-10]. The RGS box from most of these R4 RGSs have been shown to be capable of inhibiting GPCR signaling by acting as a GTPase Activating Protein (GAP) for receptor activated GTP-bound Gα subunits. There appears to be distinct but overlapping specificity of the different RGSs for the different Gα-proteins [9,11,12]. In addition, most of the evidence suggests that the R4 RGSs cannot act as a GAP for $G\alpha_s$ subunits. Nevertheless, a number of these RGSs can block signaling from $G\alpha_s$ coupled GPCRs by acting as an effector antagonist and effectively blocking $G\alpha_s$ mediated activation of effectors such as adenylyl cyclase [13].

Individual R4 RGSs also show distinct receptor specific preferences in their ability to inhibit signaling from different GPCRs [14]. This specificity appears to be determined by the ability of the different R4 RGSs to either bind to specific subsets of GPCRs or to bind to specific GPCR binding adapter proteins [3]. In spite of the absence of definite recognizable motifs, the N-terminal non-RGS portions of these proteins have been

implicated in mediating their receptor specificity and their ability to function as effector antagonists [3,15]. Thus although R4 RGSs are small proteins, their ability to interact with a number of other proteins in addition to their interaction with Gα subunits is critical for their function as modulators of GPCR signaling [16].

Multiple protein-protein interactions are a common theme for most signal transduction cascades including GPCRs [17]. In effect, GPCRs and their associated proteins are now known to assemble into large protein complexes [18,19]. In addition to interacting with a wide range of different proteins, GPCRs also form homo- and hetero-oligomers [20]. Although RGSs are members of the GPCR containing complexes, the entire repertoire of proteins that are capable of interacting with the R4 RGSs is not known [3,16].

We have previously generated specific RGS5 anti-serum and we have shown that this protein migrates as 23 kDa protein that is abundantly expressed in cardiac and skeletal muscle [4]. Here we continue our analysis of the RGS5 protein and we report a new binding partner for RGS5. In effect, we demonstrate that RGS5 can be detected as a dimer by western blot analysis in both yeast and cultured mammalian HEK293A cells that overexpress RGS5. Using a series of other experimental approaches including the expression and analysis of GFP tagged RGS5 in yeast as well as two hybrid assays, we provide evidence that RGS5 does indeed interact with itself. We also demonstrate that formation of the RGS5 dimer may have a regulatory role since we observe that the dimer is longer lived than the monomer. Thus our results suggest that the dimerization of R4 RGSs, like RGS5, may provide an additional layer of complexity involved in regulating

GPCR signaling complexes.

4. Materials and Methods

4.1 Plasmids

To make the RGS5-GFP fusion, RGS5 was amplified using the following forward 5'-ACACTAGTCAAACAATGTGCAAAGGACTTGCAGCTTTG-3' and reverse 5'-AGAAGCTTGCTTGATTAACTCCTGATAAAATCT-3' oligos with p423GAL1RGS5 as a template [21]. The PCR product was subcloned as a *Spe*I-*Hin*dIII fragment into p426GAL1-GFP [22] to generate plasmid p426GAL1-RGS5-GFP encoding an in-frame RGS5-GFP protein fusion. The plasmids encoding the different GPCR C-tails fused to GFP were constructed by first amplifying the receptor C-tails using reverse transcribed RNA that was obtained from mouse tissues (brain, testes or heart) and the following oligos: SST_5: forward: 5'-AACTAGTGGCAAACAATGTCTGACAACTTCCGC CAGAGC-3', reverse: 5'-AGAAGCTTGCAGCTTGCTGGTCTGCATAAG-3'; β_2AR: forward: 5'-CTAGTCAAACAATGAGTCCAGATTTCAGGATTGCC-3', reverse: 5'-AAGCTTGCAGTGGCAGGTCATTTGTACTACA-3'; CB_1: forward: 5'-ACTAGTCAAACAATGAAGGACCTGAGACATGCTTTCCGC-3', reverse: 5'-AAGCTTGCAGAGCCTCGGCAGACGTGTCTGT-3'; and LPA_4: forward: 5'-ACTAGTCAAACAATGTACTTCACTCTTGAATCCTTTCAG-3', reverse: 5'-

AAGCTTGGAAGGTGGATTCCAGCATTAA-3'. The amplified products were subcloned into p426GAL1-GFP as described above for RGS5. The yeast expression plasmid p425GAL-RGS5 and the mammalian expression plasmid for RGS5 were previously described [4,21]. p426GAL1-ATG-GFP expressing GFP alone under the control of the galactose inducible *GAL1* promoter was previously described [22].

4.2 Yeast cells

The *Saccharomyces cerevisiae* strain BY4741-6055 (*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sst2Δ::G418*^{R}) was used for these studies (Research Genetics). Yeast cells were routinely grown on synthetic minimal media consisting of Yeast Nitrogen Base (YNB) containing 2% glucose supplemented with the appropriate amino acids and bases [23]. Plasmids were introduced into yeast using lithium chloride and the resultant transformants were selected and maintained by the omission of the appropriate supplements from the growth media (leucine for p425GAL1 and pACT2, uracil for p426GAL1, and tryptophan for pLexA-dir vectors). Glucose was replaced with 2% galactose and 2% raffinose to induce *GAL1* dependent expression. The semi-quantitative halo assay was used to assess the ability of RGS5-GFP to function as an RGS essentially as previously described [21,24]. Sterile filter disks containing 1000 and 3000 pmoles of α-factor, were placed onto the top of a lawn of yeast cells and the plates were subsequently incubated at 30°C for 3 to 4 days.

4.3 Mammalian cultured cells

HEK 293A cells were grown in DMEM media containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (pen-strep). The cells were transfected with pcDNA alone or with pcDNA plasmid expressing RGS5 using lipofectamine (Invitrogen).

4.4 Protein extraction and western blot analysis

Soluble protein was extracted from yeast by directly heating the NaOH treated cells to 95°C in SDS-PAGE loading buffer as previously described [22]. Soluble protein was extracted from transiently transfected HEK293A cells using ice cold lysis buffer [25]. Equal amounts of soluble protein were separated by SDS-PAGE, transferred to nitrocellulose membrane and challenged with the previously described RGS5 anti-serum [4] or the commercially available GFP or LexA anti-sera (Santa Cruz Technology). HRP-conjugated secondary anti-serum were used and signals were subsequently detected with ECL plus (Amersham Bioscience) followed by exposure to X-ray film (Kodak X-Omat). The relative expression levels were determined using Multi-Analyst software (BioRad). Unless otherwise indicated, western blots were repeated at least three times with similar results.

4.5 Two hybrid analysis

Plasmid pLexA-dir was used as the bait vector and plasmid pACT2 was used as the prey vector [26]. Human RGS5 was prepared for cloning into the prey vector by PCR amplification using the respective forward 5'-GCTTACCCATACGATGTTCCAGATTACGCTATGTGCAAAGGACTTGCAGCT-3' and reverse 5'-ACTTGCGGGGTTTTTCAGTATCTACGATTCTTACT ACTTGATTAACTCCTGATA-3' oligonucleotides. The N-terminal region of RGS5 was prepared for cloning into the bait vector by PCR amplification using the following respective forward 5'-GTTGGGGTTATTCGCAACGGCGAC TGGCTGATGTGCAAAGGACTTGCAGCT -3' and reverse 5'-TCATAAGAAATTCGCCCGGAATTAGCTGGCAGGGCCTCGTCCAGCGAGGT-3' oligonucleotides. The C-terminal RGS box regions of RGS5 was prepared for cloning into the bait vector by PCR amplification using the following respective forward 5'-GTTGGGGTTATTCGCAACGGCGACTGGCTGCAGTGGCGTGATTCCCTGGAC-3' and reverse 5'-TCATAAGAAATTCGCCCGGAATTAGCTGGTTACTACTT GATTAACTCCTGATA-3' oligonucleotides. The PCR products were subcloned by recombination in yeast using *Eco*RI digested two hybrid vectors [27]. Different combinations of the plasmids were transformed into the yeast strain DSY-1 (*MATa his3Δ200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)$_4$-HIS3 URA3::(lexAop)8-lacZ GAL4*). Freshly saturated cultures of the transformed were serially diluted and spotted onto YNB glucose agar plates with and without histidine [28].

5. Results and Discussion

5.1 Identification of an RGS5 dimer

Human RGS5 is a 181 residue protein that belongs to the R4 RGS subfamily. It has a calculated molecular weight (MW) of 20.9 kDa and we have previously demonstrated that it migrates as a single band of 23 kDa when detected by western blot analysis [4]. The same 23 kDa band was detected in yeast and HEK293A cells overexpressing human RGS5 and in tissues endogenously expressing RGS5. Here we demonstrate that in addition to recognizing the 23 kDa RGS5 protein, our affinity purified RGS5 anti-serum also recognizes proteins with apparent MWs of 87 and 42 kDa in extracts prepared from yeast cells expressing RGS5 (Figure 4.1A). The 87 kDa band was also detected in extracts prepared from control yeast cells suggesting that it represents a non-specific cross-reactive protein. In contrast, the 42 kDa protein appears to be a modified form of RGS5, since it is not seen in control yeast cells that do not overexpress RGS5 (Figure 4.1A). Because the 42 kDa protein corresponds to the approximate MW of two RGS5 proteins, we hypothesized that it may represent a RGS5 dimer.

The putative RGS5 dimer is also detected in extracts prepared from HEK293A cells that are overexpressing RGS5 (Figure 4.1B). This later result suggests that the putative dimer is not an artifact of overexpressing RGS5 in the heterologous yeast cell. Using the

same affinity purified anti-sera, we previously reported that RGS5 protein could only be detected in skeletal and cardiac muscle tissue and cell lines [4]. We were unable to detect the putative RGS5 dimer in extracts prepared from these tissues or cells because of the large number of cross-reactive proteins that were recognized by the anti-serum (not shown).

5.2 Analysis of an RGS5-GFP fusion in yeast

R4 RGSs are known to interact with other proteins [8,16]. Although the interaction of RGS5 with Gα proteins is likely to be transitory, the strength of its interaction with other proteins such as the chaperone 14-3-3 is not well known [16]. Since many yeast and human proteins share similar sizes and function, it remains possible that the putative RGS5 dimer that we observe actually represents a dimer that forms between RGS5 and another protein.

To further investigate the possibility that we observe an RGS5 dimer, we constructed an RGS5-GFP fusion gene in order to express a GFP tagged RGS5 protein in yeast. Given that the RGS5-GFP protein has a calculated MW of 47.7 kDa, we should be able to detect a 95 kDa protein if RGS5-GFP does indeed form a dimer with itself.

As a first step towards characterizing the RGS5-GFP fusion protein, we used a yeast mutant lacking its RGS-encoding *SST2* gene to determine if RGS5 retained RGS function when fused to GFP. *Sst2* yeast cells are hypersensitive to GPCR stimulation and are

consequently hyper-responsive to the growth inhibitory effects of the GPCR agonist α-factor. We and others have shown that a number of different mammalian RGSs, including RGS5, can inhibit endogenous GPCR signalling and confer an increased resistance to α-factor when expressed in yeast [5,8,21,29]. Using the halo assay, we show that cells transformed with the control plasmid p426GAL1-ATG-GFP (which expresses GFP alone) show a significant zone of no growth around the filter discs containing α-factor indicating a strong GPCR mediated response (Figure 4.2A). In contrast, expression of RGS5-GFP attenuated the growth inhibitory effects of α-factor (Figure 4.2A, bottom plate). These results indicate that RGS5, like a number of other RGSs, can be fused to GFP and retain its ability to function as an RGS [22,30,31].

Western blot analysis was then carried out using extracts prepared from yeast cells expressing RGS5 or RGS5-GFP. As seen in Figure 4.1A and 4.2B, we observe 2 specific bands in cells expressing RGS5 using the RGS5 anti-serum. The 23 kDa band represents the RGS5 monomer while we have named the 42 kDa band dRGS5 to reflect the fact that it may be a RGS5 dimer. Both of these bands are absent in cells expressing RGS5-GFP. Instead, we observe a prominent band having a calculated MW of 50 kDa, which corresponds closely to the calculated MW of 47.7 kDa for RGS5-GFP (Figure 4.2B). In addition, a band having an estimated MW of 100 kDa is also present. The size of the band, which we have named dRGS5-GFP, has the appropriate MW to represent a RGS5-GFP dimer. These results strongly suggest that the observed 42 kDa that cross-reacts with the RGS5 anti-sera is indeed an RGS5 dimer.

GFP has been reported to be capable of self-dimerizing under some conditions [32,33]. In order to rule out the possibility that the observed RGS5-GFP doublet is due to

GFP itself, we performed a western blot analysis using protein extracts prepared from yeast transformants expressing GFP alone or GFP fused to the C-terminal regions of a number of different GPCRs (Figure 4.2C). Using specific GFP anti-serum, a single band corresponding to the appropriate MWs of GFP or the GFP fusions was detected (Figure 4.2C). Thus GFP does not form a dimer that is detectable by western blot analysis when expressed in yeast.

5.3 RGS5-RGS5 interaction by two-hybrid analysis

Although the previous results clearly indicate that RGS5 can form a dimer, it remains that this dimer may be a methodological artifact. We therefore made use of the yeast two-hybrid system as a different approach to determine if we could detect RGS5-RGS5 interaction [26]. Since the yeast strain used has a LexA-*HIS3* fusion reporter gene, histidine prototrophy indicates that a given bait and prey are physically interacting. The spot assay was therefore used to assess growth in the absence of histidine of yeast cells transformed with different combinations of bait and prey vectors that express the different protein fusions shown in Figure 4.3A. As a control, we show that all yeast transformants are capable of growth when the growth media is supplemented with histidine (Figure 4.3A). As a positive control, we made use of the fact that WIPI49 has been previously shown to interact with the N-terminus of sphingomyelin synthase (unpublished observation).

Yeast cells harbouring the bait plasmid expressing $SMS1^{1-70}$ [28] and the WIPI49

expressing prey vector are positive for growth in the absence of histidine (Figure 4.3A). In addition to these plasmids, we transformed yeast with different bait plasmids expressing the N-terminus ($RGS5^{1-59}$) or the C-terminal RGS box ($RGS5^{60-181}$) of RGS5 and prey vector expressing the entire RGS5 coding sequence. Of the different combinations tested, only the RGS5 box and RGS5 were found to interact (Figure 4.3A). Given that RGS5 did not interact with $SMS1^{1-70}$ or that the RGS5 box did not interact with WIPI49, the observed RGS5 Box interaction with RGS5 appears to be specific (Figure 4.3A). It nevertheless remained possible that the negative results demonstrating that the N-terminus does not interact with RGS5 is due to the $RGS5^{1-59}$ bait protein not accumulating in yeast cells. We therefore used western blot analysis to show that all the bait proteins used in this study, including the RGS5 N-terminal-LexA fusion accumulate in yeast cells (Figure 4.3B). Taken together these results provide strong evidence that RGS5 is capable of self-interacting.

5.4 RGS5 dimer is more stable than the RGS5 monomer

The levels of many of the R4 RGSs, including RGS5, are regulated in part by post-translational mechanisms [8,10,34-36]. We therefore wanted to examine the possibility that the RGS5 dimer may represent a sub-population of RGS5 proteins that are differentially regulated than the monomer. To examine the stability of RGS5, HEK293A transfected with pcDNA-hRGS5 were treated with cycloheximide and samples were harvested at intervals for western blot analysis using RGS5 specific anti-sera. Upon

blocking protein synthesis with cycloheximide, the levels of RGS5 monomer were found to decrease with a half life of 20 hours (Figure 4.4). In contrast, the RGS5 dimer remained stable with 100% of the protein still present 24 hours after the addition of cycloheximide (Figure 4.4). This demonstrates that RGS5 dimer is differentially regulated than the monomer.

5.5 Discussion

Members of the R4 RGS sub-family are best known as proteins that can serve as GAPs for subsets of different heterotrimeric Gα proteins [8-12,37]. Our understanding of their involvement in cellular processes such as their ability to function as effector antagonists as grown alongside our understanding of the diversity of proteins that interact with the R4 RGSs [13,16]. The list has grown to include chaperones, GPCRs, effectors and adapter proteins. Here we add to this list by providing the first report that describes the dimerization of a member of the R4 RGS sub-family.

RGS5 dimerization was first detected as a protein having the MW of 2 RGS5 proteins by western blot analysis in extracts prepared from both yeast and HEK 293 cells overexpressing RGS5 (Figure 4.1). Detection of a RGS5-GFP dimer in the extracts prepared from yeast cells expressing a functional RGS5 cDNA tagged with GFP served to confirm that RGS5 is capable of dimerizing (Figure 4.2). The ability of the RGS5 and RGS5-GFP dimers to remain intact after SDS-PAGE suggests that the observed RGS5-

RGS5 dimer is resistant to detergent and reducing agents that are used to prepare the samples. Although this may reflect covalent attachment, non-covalent interaction may also lead to such strongly attached dimers. The situation is reminiscent of other non-covalently attached dimers, such as GPCRs, that have been shown to remain attached under such stringent conditions [38]. The nature of the RGS5-RGS5 dimer remains at present unknown.

Yeast two hybrid analysis was used to confirm that that RGS5 was capable of self-interacting (Figure 4.3). Our results suggest that the RGS5 interacting region is present within the RGS box. These results are somewhat surprising since the RGS box is thought to be the site of interaction with Gα-proteins while the N-terminal region has been shown more versatility since it can interact with a number of different proteins including GPCRs, effectors and some adapter proteins [3,16]. The involvement of the RGS box suggests that RGS5-RGS5 interaction may have a regulatory role. For example a number of R4 RGSs interact with 14-3-3 and this interaction may serve to inhibit RGS GAP activity [16]. It would be interesting to determine if RGS5 dimerization serves a role in modulating the GAP activity of RGS5.

We previously demonstrated that RGS5 protein is abundantly expressed in cardiac and skeletal muscle [4]. Basal levels of RGSs are known to limit GPCR responsiveness while decreases in the basal levels of RGSs increase GPCR responsiveness [8,10]. Alterations in the activity of RGS5 by dimerization may therefore serve to regulate a subset of the large number of GPCRs that are known to be expressed in cardiac and skeletal muscle [39,40].

Analysis of the stability of RGS5 ectopically expressed in HEK293 cells suggested that the RGS5 monomer has a shorter half-life than the dimer (Figure 4.4). These results are consistent with previously published reports showing that RGS5 has a relatively short half-life [34-36]. The N-end rule ubiquitin dependant pathway has been shown to be involved in degrading RGS5 [35,36]. Thus dimerization of RGS5 appears to prevent the rapid degradation of RGS5 by as of yet unknown process that may simply involve the masking of the N-terminal region of RGS5. RGS5 levels, as observed for a number of other RGSs, are known to be increased in response to GPCR stimulation [4,8,12]. We nevertheless find no difference in the levels of the RGS5 monomer in HEK cells overexpressing RGS5 that are stimulated with GPCR agonists (data not shown). Although the differential stability of the monomer versus the dimer is consistent with our results, it nevertheless remains possible that the RGS5 monomer is not lost but instead it could be converted to the dimeric form. Experiments using proteosome inhibitors are underway in order to further examine these possibilities.

Although the possible significance of RGS dimerization is not known, it is worthwhile mentioning that the significance of GPCR dimerization was not well understood when it was first reported [38]. Nevertheless, today it is quite clear that GPCR-GPCR interaction is a widespread phenomenon that plays critical roles in multiple aspects of the biology of these receptors [20]. The implications for RGS5 function in regulating GPCR signalling as well as the possible dimerization of other R4 RGSs will be questions of great interest. In this context, it is interesting to note that purified RGS4 appears to form a dimer in vitro [35]. Although RGS4 and RGS5 along with RGS8 and RGS16 may be a subfamily R4 RGSs [21], these results suggest that RGS dimerization may be a common phenomenon.

6. Acknowledgements

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7. Figure Legends and Figures

Figue 4.1 Detection of a RGS5 dimer in cells overexpressing RGS5.

A. Western blot analysis of protein extracts prepared from control (Ctrl.) yeast cells or from yeast cells expressing RGS5 (RGS5). **B.** Western blot analysis of protein extracts from HEK293A cells. Cells were transfected with either the mammalian expression plasmid pcDNA (Ctrl.) or a pcDNA expressing a hRGS5 cDNA (RGS5). As a control, 0.5μg of protein extract from yeast cells expressing hRGS5 was included on the gel (left lane). In both **A** and **B**, the extracts were challenged with RGS5 anti-serum. In addition the location of bands corresponding to the sizes of RGS5 and putative RGS5 dimers (dRGS5) are indicated. A non-specific band of approximately 87 kDa is apparent in the extracts prepared from both mammalian and yeast control cells and is indicated by an "*". Similar results were obtained in four different experiments.

Figure 4.2 Analysis of a GFP tagged RGS5 gene in yeast.

A. Halo assays were performed on yeast transformants harbouring plasmid p426GAL1-ATG-GFP (GFP) or p426GAL1-RGS5-GFP (RGS5-GFP). Sensitivity to the growth inhibitory effects of the yeast GPCR agonist is proportional to the zone of no growth surrounding the α-factor containing filters. **B.** Western blot analysis using RGS5 anti-serum with extracts prepared from yeast cells expressing RGS5 or RGS5-GFP. The locations of the RGS5 and RGS5-GFP monomers and the putative dimers (dRGS5 and

dRGS5-GFP) are shown. An "*" denotes the non-specific 87 kDa band while a double asterisks (**) denotes a band that is reproducibly observed but whose origin remains unknown. Molecular weight markers in kDa are shown on the right of the blot. **C.** Western blot analysis, using GFP anti-sera, of extracts prepared from yeast cells expressing GFP alone and GFP fused to the C-tails of different GPCRs (SST_5, somatostatin receptor 5, 56 residues, GenBank accession No. NM001053; β_2AR, β_2-adrenergic receptor 2, 90 residues, accession No. BC032883; CB_1, cannabinoid receptor1, 70 residues, accession No. NM007726; and LPA_4, lysophosphatidic acid receptor 4, 57 residues, accession No. NM020028). All western blots were performed at least three times with similar results.

Figure 4.3 Analysis of RGS5-RGS5 interaction using the yeast two hybrid system.

A. A schematic diagram depicting the fusion proteins used in this study is shown at the top. Yeast cell were transformed with different combinations of bait (BAIT) and prey (PREY) expressing plasmids as indicated. Cultures of the different transformants were serially diluted and aliquots were spotted onto nutrient agar with (+His) and without (-His) histidine. $SMS1^{1-70}$ represents the N-terminal 70 residues of sphingomyelin synthase (GenBank accession No. AY509044) and WIPI49 is a two hybrid clone that was found to interact with $SMS1^{1-70}$ in a two hybrid screen of a mouse cardiac cDNA library (Dualsystems Biotech AG). Growth in the absence of histidine (-His) indicates that an interaction is occurring between the bait and the prey tested. Similar results were obtained in 3 different experiments using two different groups of yeast transformants. **B.**

Extracts prepared from yeast cells expressing the different LexA-bait protein fusions, described in A, were analyzed by western blot using a specific LexA anti-serum.

Figure 4.4 Analysis of the stability of the RGS5 monomer and dimer.

Identical cultures of HEK293A cells expressing hRGS5 were treated with cycloheximide (CHX, 50μg/ml). Samples were harvested prior to the addition of CHX (0) and after 4, 8, and 24 hours (hr). Extracts were prepared and analyzed for RGS5 expression by western blot. Similar results were obtained in two independent experiments.

Figue 4.1 Detection of a RGS5 dimer in cells overexpressing RGS5.

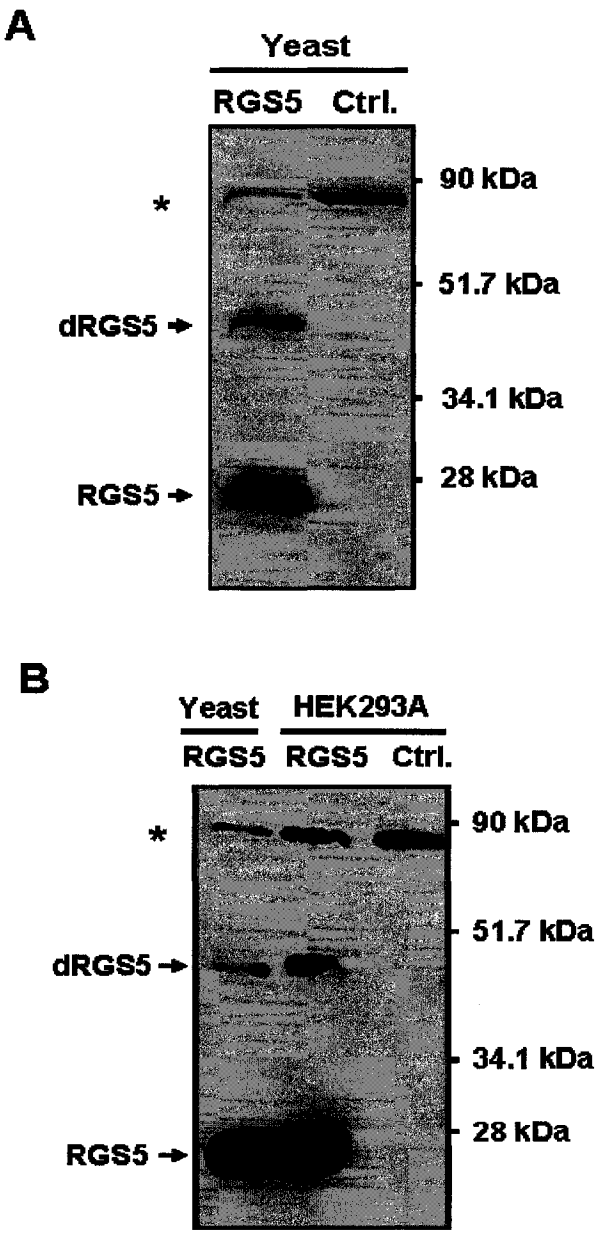


Figure 4.2 Analysis of a GFP tagged RGS5 in yeast.

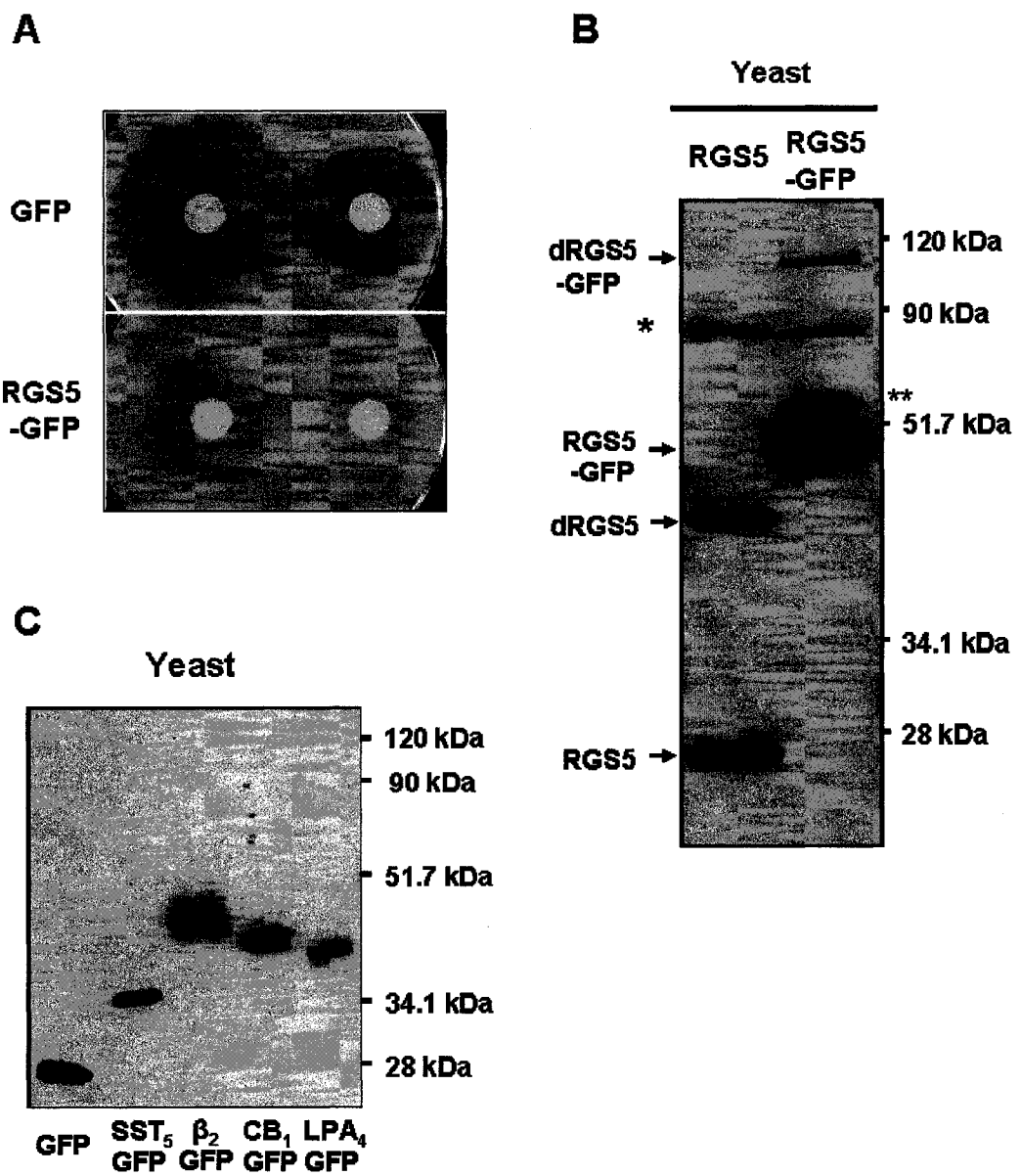


Figure 4.3 Analysis of RGS5-RGS5 interaction using the yeast two hybrid system.

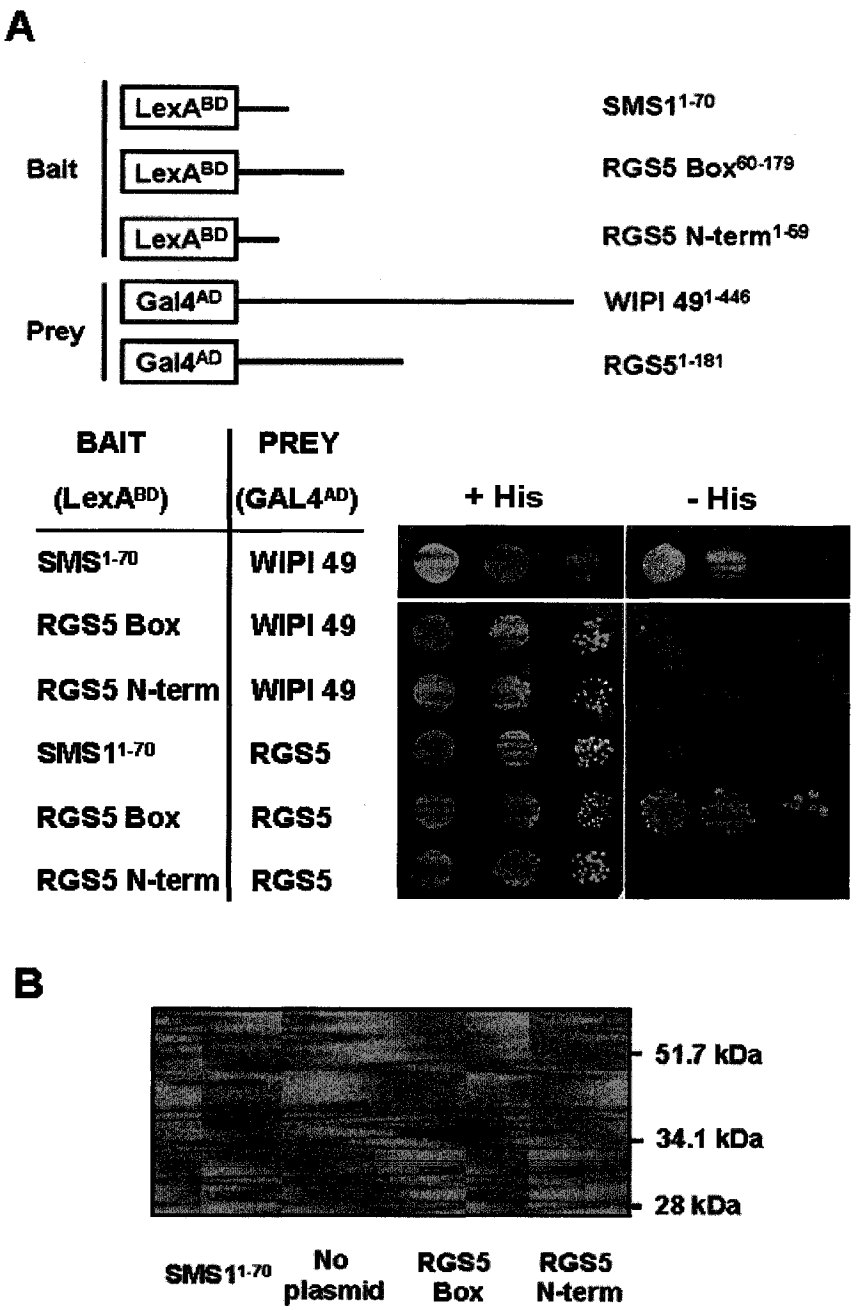
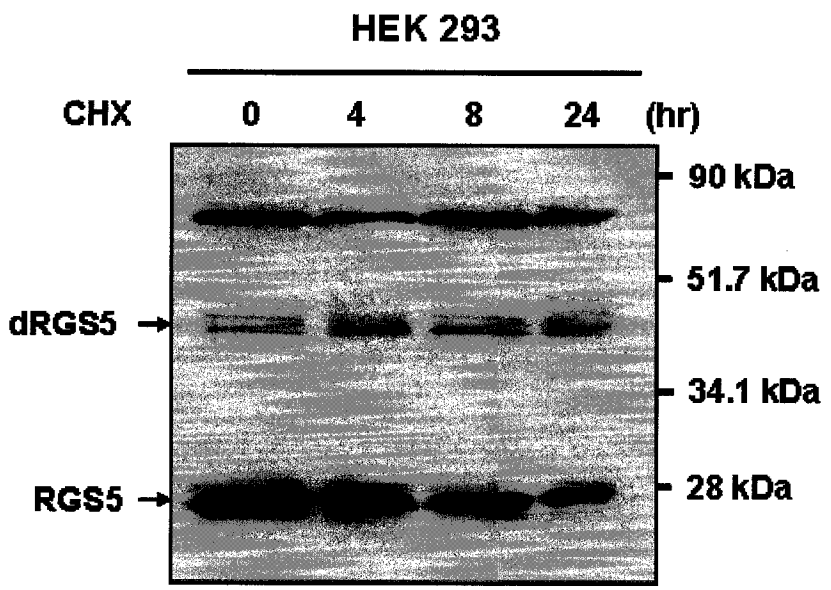


Figure 4.4 Analysis of the stability of the RGS5 monomer and dimer.



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Chapter 5 Summary and General Discussion

1. SMS1 is a newly cloned gene, encoding an enzyme important for sphingolipid metabolism.

Sphingomyelin synthase (SMS) has been known as an intracellular ceramide regulator for a long time [1]. Although Luberto et al. [2] identified bacterial SMS in 2003, the human SMS cDNA sequence was only published in 2004 [3]. I screened mouse T cell cDNA library and successfully cloned sphingomelin synthase 1 (SMS1) [4] based on its ability to prevent human RGS1 mediated cell growth arrest in yeast [5]. My observation that SMS1 also prevents Bax induced cell death in yeast provided evidence that SMS1 may be anti-apoptotic. The following different stimuli, including H_2O_2, high concentration of NaCl and high temperature, further confirm the anti-apoptotic characteristics of SMS1 [4]. My hypothesis that SMS1 suppresses ceramide production leading to anti-apoptosis is supported by other studies [6-8].

Ceramide is a key metabolite in the sphingolipid metabolism pathway [9]. Many researchers report that up-regulation of intracellular ceramide level in cells is associated with a variety of apoptotic stimuli [10-13]. Ceramide has also been widely reported to be involved in a number of pathologies, such as insulin resistance and lipotoxicity [14],

infectious diseases [15,16] and cell death mediated by some cancer therapies [10,17-19].

In addition to the *de novo* pathway, ceramide can also be generated by hydrolysis of sphingomyelin (SM), glucosylceramide (GlcCer), galactosylceramide (GalCer), ceramide-1-phosphate (Cer-P), and/or sphingosine (SPH) [19,20]. A number of other enzymes, such as ceramidases, which are involved in degrading ceramide, are also anti-apoptotic [17,18]. The recent cloning of SMS1 and SMS2 represents a major step towards increasing our understanding of the mechanism involved in regulating ceramide metabolism [21,22].

Another second messenger, ROS, is known to be elevated in response to a wide variety of different stresses as well as ceramide. Both are involved in the apoptosis induction. One interesting question waiting to be answered concerning ROS and ceramide is which one is activated first? My experimental results show that SMS1 can make cells survive from both ROS and ceramide induced cell death. From the literature, currently two conflicting directions exist together. In leukemia cells, anticancer agent N-(4-hydroxyphenyl)retinamide (4-HPR) induces a increase in ceramide levels by sphingomyelin hydrolysis first, and then by *de novo* synthesis. This ceramide accumulation is responsible for early ROS generation [23]. However, other reports said that ROS activates either neutral or acidic sphingomyelinase to generate ceramide [24,25]. In addition, it is also possible that ROS and ceramide activation belong to two different pathways, but cross-talk happens back and forth between these 2 different pathways.

As a useful model system, yeast, nevertheless, does not contain SMS, but instead, it has a functional homologue enzyme, inositol phosphorylceramide (IPC) synthase [9].

This IPC synthase, encoded by *AUR1*, converts phytoceramide to IPC, and further decreases the accumulation of another cytotoxic phytosphingolipid, phytosphingosine (PHS) [26]. My data has shown that AUR1-C expressing yeast cells survive from 1M NaCl mediated growth arrest. This is similar to what we observed in yeast cells expressing SMS1, indicating that SMS1 and AUR1 are both involved in the yeast apoptosis pathway [4].

2. Alternative splicing and apoptosis

Our analysis of the mouse SMS1 gene revealed that it is alternatively spliced to produce 4 different mRNAs (SMS1α1, SMS1α2, SMS1β and SMS1γ) that have distinct tissue distributions [27]. Osada's group recently reported the cloning of SMS1 from a pig liver cDNA library [28]. They called their sequence SMS1α2 based on our nomenclature [27]. This indicates that our nomenclature is likely to be adopted by the scientific community.

A recent review on SMS1 acknowledged our report showing that the mouse SMS1 gene is alternatively spliced. They unfortunately had no new insight into the possible role of alternative splicing of SMS1 [29]. A good deal more work, such as the biology functional expression in mammalian cells, will be required to understand the function of the different SMS1 proteins.

The 4 SMS1 transcripts were also found to produce 3 different proteins (SMS1α,

SMS1β and SMS1γ). The biochemically active SMS1α protein consists of 413 residues [3,27]. The other 2 SMS1 proteins, SMS1β and SMS1γ, produced by alternative splicing are C-terminally truncated SMS1 proteins that are unlikely to have SMS1 sphingomyelin synthase activity [3,29]. All SMS1 proteins contain a sterile alpha motif (SAM) at their N-terminus [30,31]. Because SAM containing proteins can interact each other via their SAM domains, we developed and tested the hypothesis that the truncated SMS1 proteins serve to regulate sphingomyelin synthase activity [27].

Alternative splicing can be regarded as another process of regulating gene expression [32], because it allows the exons of primary transcripts (pre-mRNAs) from the same gene to produce discrete protein isoforms that may confer distinct properties and fulfill different functions [32,33]. For example, plasma membrane Ca^{2+} ATPases (PMCAs) are essential to maintain intracellular Ca^{2+} homeostasis [34]. Different PMCA isoforms either help control slow, tonic Ca^{2+} signals in some cells or rapid, efficient Ca^{2+} extrusion in others. Recent studies suggest that alternatively spliced regions in the PMCAs are responsible for their unique targeting, membrane localization, and signaling cross-talk [35].

Alternative splicing also involves in regulation of apoptosis [36-39]. As an example, the long version of the transcript from the Bcl-x gene, Bcl-x_L, contains all 4 BH domains and is anti-apoptotic. While the short version of Bcl-x, Bcl-x_S, lacks BH1 and BH2 domains and is apoptotic by serving to antagonize the functions of the anti-apoptotic Bcl-2 and Bcl-x proteins [32].

3. RGS5 dimerization

The existence of GPCR dimers has been suspected for more than 20 years [40]. Dimerization gradually gained acceptance as a general feature of GPCRs since the study of the crystal structure of rhodopsin revealed the existence of homodimers [41]. The application of new technologies like fluorescence resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET), which have served to demonstrate protein dimerization in living cells have now been widely used to show that GPCRs oligomerize [41-43]. GPCR dimers have been proved to represent the operative units to achieve optimal coupling with G proteins [44] and to reach the cell surface [43].

However, RGS dimerization itself has never been reported before. Here, we have found evidence that RGS5 can dimerize using RGS5 anti-serum that we have previously developed [45]. Since our paper is now in press, there have been as of yet no published papers that have commented on the possible function of the dimerization of RGS5 or of other RGSs. It therefore goes without saying that a good deal of work is still needed to discover the possible function of RGS5 dimer. For instance, my data of RGS5 dimers are more stable than the monomers suggests that the dimer may be required for RGSs to be a GAP. Based on this point, RGSs dimerizations, either homo- or hetero-dimers, are also valuble in being checked, especially between those R4 subfamily members, RGS1, 4, 8, 16 etc.

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Appendices

Appendix I. Copyright permissions

Appendix II. Published papers

oxygen species (ROS) as well as the involvement of the mitochondria and possibly cytochrome *c*. Yeast also contains a number of orthologues to important mammalian apoptotic genes, including AIF (*AIF1*), caspase (*YCA1*), OMI/Htr2A (*NMA111*), DJ-1 (*HSP31*), as well as a nuclease (*TAT-D*) that is a strong candidate to be involved in DNA degradation associated with cell death (Madeo *et al.*, 2002; Fahrenkrog *et al.*, 2004; Wilson *et al.*, 2004; Wissing *et al.*, 2004; Qiu *et al.*, 2005). Overexpression of the yeast apoptotic proteins can serve to initiate or enhance cell death, while yeast strains lacking any of these genes show a decreased response to a number of different death stimuli.

Although yeast does not contain Bcl-2-like proteins, the heterologous expression of proapoptotic Bax or Bak in yeast serves to induce death. Numerous studies suggest that Bax-mediated cell death in yeast is mechanistically similar to the process that occurs in mammalian cells (Priault *et al.*, 2003; Gonzalvez *et al.*, 2005). Numerous groups have used inducible promoters to generate yeast cells that have conditionally lethal Bax-dependent phenotypes as a system to screen heterologous cDNA libraries and identify novel antiapoptotic sequences. The sequences identified represent a variety of different proteins, including DNA binding proteins (HMGB1), prion protein (PrP^C), enzymes involved in inhibiting free radicals such as peroxidases, Bax-binding proteins such as BI-1 and Ku70, a plant VAMP protein involved in vesicular trafficking, as well as a variety of proteins of unknown function (Xu & Reed, 1998; Greenhalf *et al.*, 1999; Kampranis *et al.*, 2000; Levine *et al.*, 2001; Pan *et al.*, 2001; Moon *et al.*, 2002; Brezniceanu *et al.*, 2003; Sawada *et al.*, 2003; Li & Harris, 2005). In spite of numerous reported screens, relatively few known antiapoptotic sequences have been identified. Here we screened mouse T-cell and human heart cDNA libraries to identify sequences capable of suppressing the growth-inhibitory effects of Bax in yeast. We report that a mouse sphingomyelin synthase 1 (SMS1) cDNA suppresses the negative effect of Bax expression on yeast growth. We further demonstrate that SMS1 not only suppresses the effects of Bax overexpression, but it also blocks the deleterious effects of a number of other stresses, including hydrogen peroxide, osmotic stress, elevated temperature and exogenously supplied sphingolipids. Given that SMS1 uses sphingolipid ceramide as a substrate to synthesize sphingomyelin (Hannun & Luberto, 2004), our results suggest that SMS1 protects against cell death by reversing the stress-inducible increase in the levels of proapoptotic ceramide.

Experimental procedures

Yeast strains and plasmids

Strain BY4741 (*MAT***a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) was used throughout this study. A mouse Bax cDNA cloned into the *HIS3*-containing vector pGILDA was used to express Bax under the control of the *GAL1* promoter. The *AUR1*-C sequence was amplified by PCR using pYC070 (Hansen *et al.*, 2003) as a template and the following forward 5′-ACTAGTGGATCCCCCGGGCTGCAGGAATTCGATTGCG TATGGCAAACCCTTTTTCG-3′ and reverse 5′-CATGGTG GCGATGGATCCCGGGCCCGCGGTACCAGCCCTCTTTA CACCTAGTGACGT-3′ oligonucleotide primers. The *AUR1*-C PCR product was subcloned in frame with GFP using *Spe*I and *Hin*dIII into plasmid p425GAL1-GFP to make p425GAL1-*AUR1*-C-GFP (Somerville *et al.*, 2003). The GFP-expressing plasmid p425GAL1-ATG-GFP was used as control for the expression of *AUR1*-C-GFP in yeast.

Yeast growth and transformations

Synthetic minimal media consisting of yeast nitrogen base (YNB) containing 2% glucose supplemented with the appropriate amino acids and bases was used to routinely grow yeast (Adams *et al.*, 1997). In order to induce expression of sequences under the control of the *GAL1* promoter, glucose was replaced with 2% galactose and 2% raffinose. The cDNA library, as well as individual plasmids, was introduced into yeast using lithium acetate. The resultant transformants were selected and maintained by omitting the appropriate nutrient from the growth media.

Screening of mammalian cDNA libraries in yeast

The mouse T-cell cDNA library was cloned into the galactose-inducible pYES2 yeast expression vector, while the heart cDNA library was cloned into the galactose-inducible pYES-DEST52 vector (Perier *et al.*, 1994). Yeast cells harboring pGILDA-Bax were transformed to uracil prototrophy using plasmid DNA from the cDNA libraries. The transformants were directly plated onto YNB nutrient agar containing galactose, and allowed to grow for 5–6 days at 30 °C. Cells expressing Bax give rise to smaller colonies. A total of *c.* 300 000 transformants from the T-cell library and 900 000 from the cardiac cDNA library were screened for suppressors of Bax by identifying colonies that have increased in size. Large colonies were purified by replating onto fresh YNB nutrient agar media containing galactose. Plasmid DNA was subsequently isolated from the transformants that gave rise to large colonies after purification. These plasmids were then reintroduced into yeast cells harboring pGILDA-Bax and their colony size, after 3–4 days of growth at 30 °C, was compared to control cells expressing Bax alone. The nucleotide sequence of the clones that still gave rise to large colonies were then determined. A total of 161 positive clones were identified in the primary screens, and 82 of these turned out to suppress the growth-inhibitory effects of Bax when re-introduced into naïve cells.

Cell growth and viability assays

The spot growth assay was used to assess the ability of different yeast transformants to grow under different conditions. Briefly, freshly saturated glucose cultures of the different transformants were serially diluted (1–5) and 10 μL of each dilution was directly spotted on nutrient agar plates containing either glucose or galactose and subsequently incubated at 30 °C for 3–5 days. In order to ensure that an equal number of cells were used, cell numbers for the different cultures were made equal in the first dilution using the spectrophotometrically (600 nm) measured cell densities. For all spot assays shown, identical results were obtained in at least three independent experiments. Unless otherwise indicated, all cells were grown at 30 °C. Viability was determined by microscopical examination of cells stained with the vital dye trypan blue (Wright *et al.*, 1999). Cells were stained with 0.1% trypan blue for 5 min and at least 300 cells were scored for each time point.

The viability of cells expressing Bax in the absence and in the presence of SMS1 was also determined by the colony formation assay. This assay was performed by using freshly saturated glucose-grown cells to inoculate galactose-containing media. The cells were grown for 18–24 h, cell number was determined microscopically, and after serial dilution 300 cells were plated on nutrient agar and incubated for 2–3 days at 30 °C and the resultant colonies were counted. At least three plates were scored per experiment. For growth curves, freshly saturated cultures grown in YNB glucose-containing media were diluted into fresh galactose-containing YNB media. The cultured cells were continuously incubated at 30 °C, aliquots were removed at different times and cell density was determined spectrophotometrically (OD_{600}). The effect of ceramide on cell growth was determined essentially as described (Fishbein *et al.*, 1993). Briefly, 2 mL of YNB galactose media were inoculated at low cell density (1.5×10^4 cells mL^{-1}) using freshly saturated cultures of yeast cells harboring pYES-SMS1 or the empty vector. The cultures were grown with increasing concentrations (0 to 20 μM) of the cell-permeable ceramide analog C_2-ceramide (CalBiochem, San Diego, CA). The cells were incubated overnight and growth was determined by measuring the density of the cultures at 660 nm. An $OD_{660\,nm}$ between 0.3 and 0.5 was routinely observed in the control cultures grown without ceramide.

Analysis of the effect of the Bax suppressors on *GAL1* promoter function

Plasmid p425GAL1-ATG-GFP, which expresses the GFP under the control of the *GAL1* promoter, was used as a reporter to monitor the effect of different Bax suppressors to interfere with the galactose-mediated induction of the *GAL1* promoter (Somerville *et al.*, 2003). Cells containing p425GAL1-ATG-GFP were transformed with either empty vector or with seven different Bax suppressors identified in the screen. Freshly saturated glucose-grown cultures of these transformants were used to inoculate fresh galactose-containing media (1 in 20 dilution) and the cells were allowed to grow for 6 h. Soluble protein was extracted from 2 mL cultures by NaOH treatment and boiling in SDS loading buffer as previously described (Somerville *et al.*, 2003). The extracts were separated by SDS-PAGE and analyzed by Western blot using a specific GFP antiserum as previously described (Somerville *et al.*, 2003). Protein loading was equalized by prior densitometric analysis of coomassie-stained gels. Yeast cells harboring a plasmid expressing somatostatin receptor 5 intracellular loop 3-GFP fusion gene, p425GAL1-SST5i3-GFP, was used as a positive control for the GFP Western.

Results and discussion

Identification of multiple Bax suppressors

A large number of mammalian antiapoptotic proteins have been described, but relatively few of these proteins have been reported as being able to suppress Bax- mediated cell death in yeast. We therefore reasoned that the strategy of functionally screening mammalian cDNA libraries in yeast cells expressing Bax would likely lead to further identification of novel mammalian anti-apoptotic genes. By screening a T-cell and a heart cDNA expression library in yeast expressing Bax, we identified 82 clones that were capable of suppressing the growth-inhibitory effects of mouse Bax in yeast. The identity of these clones was revealed by sequence analysis and searching the GenBank database. We found that the clones fell into several functional groups: 16 mitochondrial, six cytoskeletal, six protein synthesis, six DNA/chromatin, two RNA binding, seven stress/ubiquitin, two vesicular trafficking, one metabolism, one signal transduction, one sphingolipid enzyme, 14 largely uncharacterized genes and 20 that were represented more than once (not shown). The functional heterogeneity of the Bax suppressors identified here is in agreement with the observation that Bax mediates pleiotropic effects leading to death in yeast (Priault *et al.*, 2003; Belhocine *et al.*, 2004; Dimitrova *et al.*, 2004; Reekmans *et al.*, 2005). Although many of the clones corresponded to known anti-apoptotic sequences such as Hsp72, only one of the clones, corresponding to HMGB1, has previously been identified as being a suppressor of Bak in yeast (Brezniceanu *et al.*, 2003).

Most cDNA library screens carried out to identify Bax suppressors in yeast identified a number of clones capable of suppressing Bax (Pan *et al.*, 2001; Moon *et al.*, 2002; Chen *et al.*, 2004). Given that *GAL* promoters were used to express Bax, the ability of the different suppressors to reduce *GAL1*

promoter expression was examined to make sure that the different suppressors do not interfere with the galactose-mediated induction of Bax. We therefore used a *GAL1*-GFP reporter gene to monitor the effect of a subset of different clones on the levels of galactose-induced expression of GFP. Western blot analysis was carried out using extracts prepared from cells harboring the GFP-expressing plasmid p425GAL1-ATG-GFP (Somerville *et al.*, 2003). The levels of GFP detected in cells containing the GFP reporter gene and the control plasmid (Fig. 1, vector) was similar to the amount of GFP found in cells harboring clones 1, 2, 3, 5 and 7 (Fig. 1). This suggests that at least a subset of our Bax-suppressing clones do not interfere with *GAL1*-mediated expression and are likely specific for Bax. Cells expressing clones 4 and 6 had lower levels of GFP, suggesting that these clones may interfere with *GAL1* promoter function and may therefore not represent true Bax suppressors. This also suggests that a subset of the 61 clones identified as Bax suppressors may be false positives. For the reasons enumerated below, clone 1, which did not reduce *GAL1*-dependent expression of GFP, was chosen for further study.

Characterization of mouse SMS1 as a suppressor of Bax-mediated growth inhibition in yeast

The complete nucleotide sequence of one of the suppressors (clone 1 in Fig. 1) revealed a 2364-bp cDNA (GenBank accession no. AY509044) with a predicted open reading frame of a 413-residue protein that was found to be 97% identical to the recently identified human SMS1 (Huitema *et al.*, 2004). A number of previous reports have suggested that SMS is likely to be an antiapoptotic protein, but the contribution of the recently cloned SMS1 and SMS2 has not been examined. As a first step towards characterizing SMS1, yeast cells were transformed with the plasmids pGILDA-Bax or pYES-SMS1, either alone or in combination. The transformants were grown overnight in liquid glucose-containing media and serial dilutions were spotted onto glucose-and galactose-containing nutrient agar plates. Since both the SMS1 and Bax cDNAs are expressed under the control of the yeast *GAL1* promoter, there was no difference in the growth of the different transformants on glucose-containing plates (Fig. 2a, left panel). In contrast, yeast cells containing the galactose-inducible mouse Bax cDNA failed to grow to any significant extent on galactose-containing plates, when compared to yeast cells that contain control plasmids (Fig. 2a, right panel). Overexpression of SMS1 alone had no detectable effect on growth, while cells co-expressing SMS1 and Bax showed a significant increase in growth (Fig. 2a, right panel). We also used growth curves to monitor the effect of SMS1 on the growth of Bax-expressing yeast cells. Glucose-grown cells were inoculated into fresh galactose media and the growth of the cells was subsequently monitored over time at 30 °C. Yeast cells expressing Bax alone showed a significant decrease in the rate of growth and the final cell density reached, when compared to control cells harboring empty vectors (Fig. 2b). The expression of SMS1 was found to enhance both the growth rate and the final culture density of cells expressing Bax.

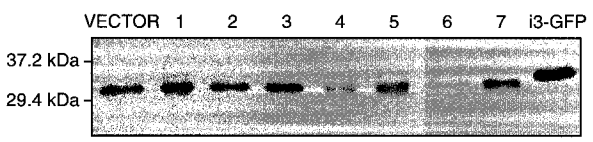

Fig. 1. The effect of Bax suppressors on the expression of a *GAL1*-GFP reporter gene. An equal amount of protein extracts prepared from galactose-grown yeast cells harboring p425-GAL1-GFP and empty vector (Vector) or different Bax suppressor clones (lanes 1–7) were analyzed by western blot analysis using a GFP-specific antiserum. A single 34 kDa band corresponding to GFP was detected (Somerville *et al.*, 2003). Extracts prepared from cells expressing a 36.7 kDa somatostatin receptor 5 intracellular loop 3 fused to GFP (i3-GFP) served as a positive control. Molecular-weight markers (in kDa) are shown on the left.

To examine the possibility that SMS1 may also be preventing Bax-mediated cell death, we examined the viability of yeast cells expressing different plasmids. Freshly saturated glucose-grown cultures of the different yeast transformants were diluted into galactose-containing media and allowed to grow for 6 h. Aliquots of the cultures were removed and cell viability was then determined by microscopical examination of cells stained with the vital dye trypan blue. Viable cells exclude the trypan blue, so the ratio of blue to colorless cells was used to determine the percentage of viable cells. Yeast cells containing empty vectors or the SMS1-expressing plasmid showed greater than 90% viability in this assay (Fig. 2c). In contrast, the viability of Bax-expressing cells was reduced to 61.8%. Yeast cells coexpressing SMS1 along with Bax showed an increase in viability up to 74.3%. We also used the colony formation assay to determine the ability of SMS1 to prevent Bax-mediated cell death. In this assay we found that only 19% of Bax-expressing cells were able to form colonies. The viability of Bax-expressing cells was increased to 80% when SMS1 was co-expressed (Fig. 2d). The observed differences in viability of Bax-expressing cells in the two assays reflects the differences in time that Bax was expressed prior to determining viability and the fact that cells can exclude vital dye and appear alive, yet are unable to form colonies when plated. This can lead to an overestimation of the number of viable cells (Fannjiang *et al.* 2004). These results indicate that SMS1 can partially prevent Bax-mediated cell death. The identification of SMS1 is to our knowledge, the first case in which an enzyme involved in sphingolipid metabolism has been identified as an inhibitor of the apoptotic effects of Bax. Nevertheless, these results are consistent with

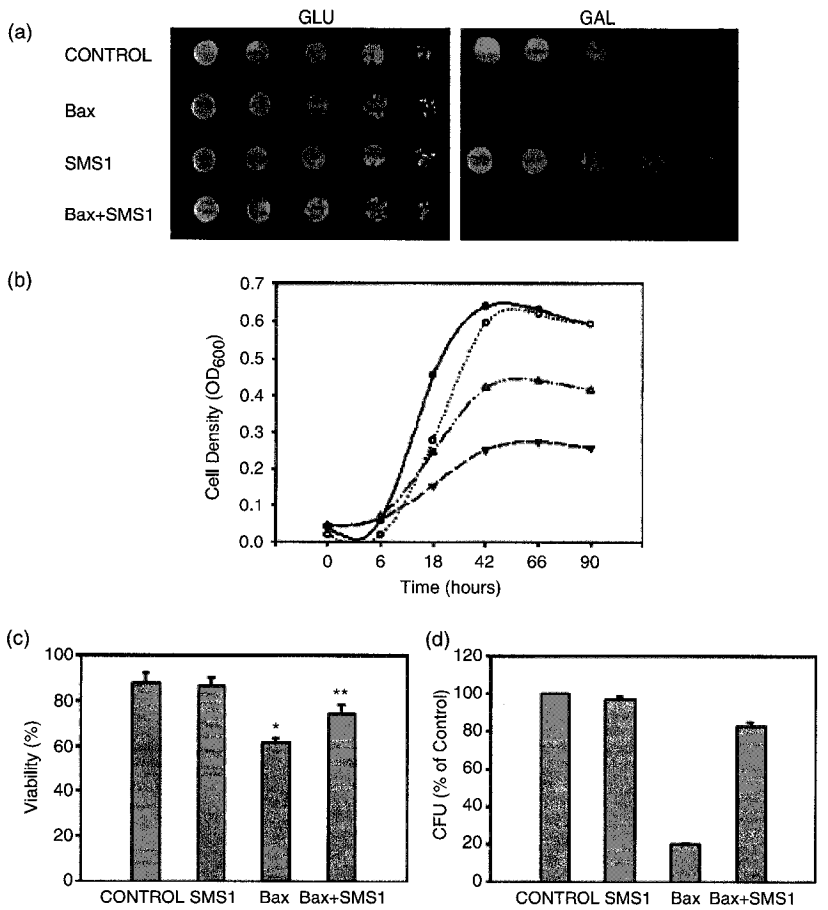

Fig. 2. SMS1 suppresses the growth-inhibitory and death-inducing effects of expressing murine Bax in yeast. The growth and viability of different yeast transformants harboring empty vector (CONTROL), Bax or SMS1-expressing plasmids were analyzed. The transformants were first grown to saturation in glucose-containing media. (a) The saturated cultures were serially diluted and 10 μL aliquots of each dilution were spotted on glucose (GLU)- and galactose (GAL)-containing nutrient agar media. Photographs of the plates after incubation at 30 °C are shown. (b) Glucose-grown cells were used to inoculate galactose-containing media and growth was monitored by removing samples and measuring the turbidity at optical density of 600 nm (OD_{600}). The following symbols denote the different strains examined: empty vector control (●), SMS1 (○), Bax and SMS1 (△) and Bax alone (▼). (c) Glucose-grown cultures were used to inoculate galactose-containing media. The cultures were allowed to grow for 6 h and cell viability was determined by microscopical examination of cells stained with the vital dye trypan blue. At least 300 cells were scored for each data point. Data represent the mean ± standard error of the mean (SEM) of three independent experiments. *,**One-way ANOVA analysis followed by an all pairwise comparison procedure (Holm–Sidak method) showed that there are significant differences with the control and SMS1 groups ($P < 0.003$). (d) Glucose-grown cultures were used to inoculate galactose-containing media. Yeast cells were then grown overnight to saturation in galactose-containing media and viable cells were determined as the percentage of cells that formed colonies on agar media. The viable cell number obtained from control cells harboring empty vectors was arbitrarily used as 100%. Data represent the mean ± standard deviation (SD) of triplicate assays and are typical of two independent experiments.

numerous other studies that have implicated sphingolipid-metabolizing enzymes, including SMS, as being antiapoptotic in mammalian cells (Meng *et al.*, 2004; Taguchi *et al.*, 2004).

SMS1 decreases cell death in response to hydrogen peroxide and high osmolarity

Although Bax expression in yeast clearly leads to cell death, some controversies nevertheless remain as to whether this process truly mimics apoptosis or some other form of programmed cell death, such as autophagy (Priault *et al.* 2003). We therefore ascertained whether SMS1 prevents cell death in response to other stimuli. ROS are common byproducts of metabolism, causing cellular damage that can also induce apoptosis. A number of chemicals, such as hydrogen peroxide (H_2O_2), are used as ROS donors to induce cell death in yeast cells (Madeo *et al.*, 1999). To test the effects of SMS1 on H_2O_2-mediated cell death, glucose-grown cultures of cells harboring either control or

SMS1-expressing plasmid were incubated in galactose media for 5 h and challenged with increasing concentrations of hydrogen peroxide for a further 5 h. A 10 μL aliquot of each culture was then spotted onto nutrient agar media containing either glucose or galactose and allowed to grow at 30 °C. Compared to the controls, cells expressing SMS1 showed enhanced ability to grow, already evident after being treated with as little as 5 mM H_2O_2 (Fig. 3a). Cells expressing SMS1 during the period of H_2O_2 treatment showed enhanced growth if plated in the presence of glucose or galactose, indicating that the expression of SMS1 was not required for the cells to recover after H_2O_2 treatment. Thus SMS1 is required to protect the cells from H_2O_2 treatment and it is not required for growth after the stress is over. We also used vital dye to ascertain the effect of SMS1 on cell viability after H_2O_2 treatment. As shown in Fig. 3b, control yeast cells showed a dose-dependent decrease in viability with increasing concentrations of H_2O_2. The negative effect of H_2O_2 on cell viability was largely abolished in cells expressing SMS1. Viability was decreased to 47% in control cells treated with 20 mM H_2O_2, while the viability of cells expressing SMS1 remained at 75% of untreated control cells (Fig. 3b).

Osmotic stress leads to growth inhibition and it can also induce cell death via apoptosis (Huh *et al.*, 2002). We therefore examined the ability of SMS1 to prevent the growth inhibitory effects of high salt. Freshly saturated glucose-grown cultures of control as well as of SMS1-containing cells were serially diluted and aliquots spotted onto nutrient media agar plates containing glucose or galactose and 6% NaCl. On galactose medium, SMS1-expressing cells showed an enhanced ability to grow on media containing 6% NaCl compared to control cells (Fig. 4a, right panel). Growth of both cells was identical on glucose media containing 6% NaCl, indicating that the same numbers of control and SMS1-expressing cells were used in the experiment (Fig. 4a), left panel). We also used trypan blue staining to monitor the effect of SMS1 on the viability of cells growing on media containing 6% NaCl. The viability of control yeast cells growing on media containing high levels of salt gradually decreased from 81% after 1 day to 46% after 3 days (Fig. 4b). In contrast, the viability of SMS1 cells showed a modest 8% decrease after 1 day of growth on high-salt media. Even after 3 days on high-salt media, the viability of SMS1 expressing cells remained high at 75% (Fig. 4b).

The results presented so far indicate that SMS1 is capable of suppressing the apoptotic effects of a number of different stimuli. DNA-damaging agents, such as ultraviolet light (UV), is another common stimulus leading to cell death that may involve apoptosis (Del Carratore *et al.*, 2002). In the absence of UV, there was no difference in the ability of control or SMS1-containing cells to grow on glucose or galactose media (Fig. 4c). Although there was a noticeable

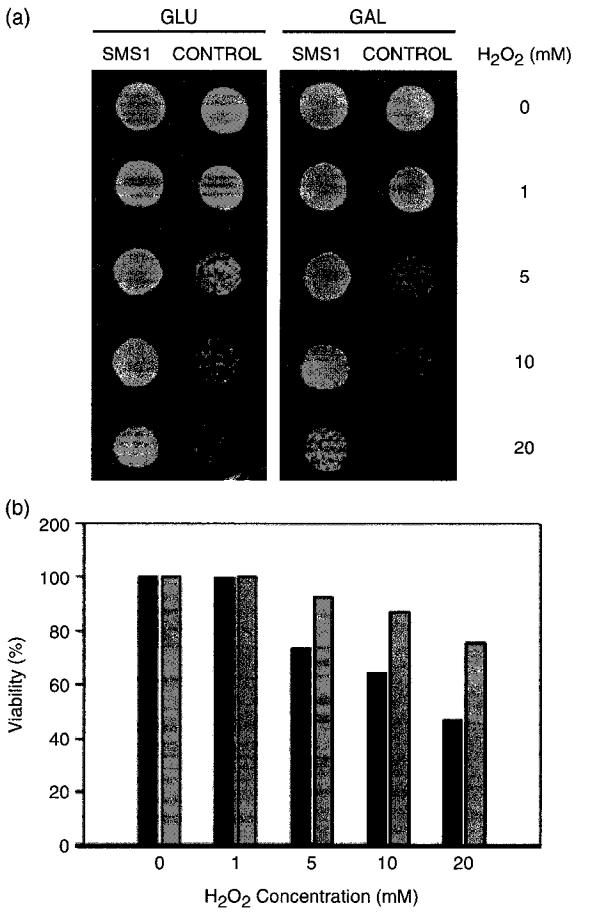

Fig. 3. SMS1-expressing yeast cells are resistant to hydrogen peroxide-mediated death. Yeast transformants harboring empty vector (CONTROL) or pYES-SMS1 (SMS1) were grown in galactose-containing media for 5 h. Identical cultures were treated with the indicated concentrations of hydrogen peroxide (H_2O_2) for 5 h. (a) 10 μL of each culture were then directly spotted onto glucose (GLU)- or galactose (GAL)-containing media and photographs of the resultant plates after incubation at 30 °C are shown; (b) viability of the H_2O_2-treated transformants harboring empty vector (black) or pYES-SMS1 (gray) was determined by microscopical examination of trypan blue-stained cells. At least 300 cells were examined for each data point and similar results were obtained in two independent experiments.

decrease in the ability of cells to grow after treatment with 10 mJ cm^{-2} UV, there was no difference in growth between cells expressing SMS1 and control cells. Similarly, SMS1 did not protect cells from lower or higher doses of UV (5 and 50 mJ cm^{-2}; not shown). These results indicate that SMS1 is not able to prevent cell death in response to all stresses.

SMS1 partially reverses the growth-inhibitory effects of exogenously supplied sphingolipids

SMS is a key enzyme in sphingolipid metabolism that utilizes ceramide and phosphatidylcholine (PC) to produce

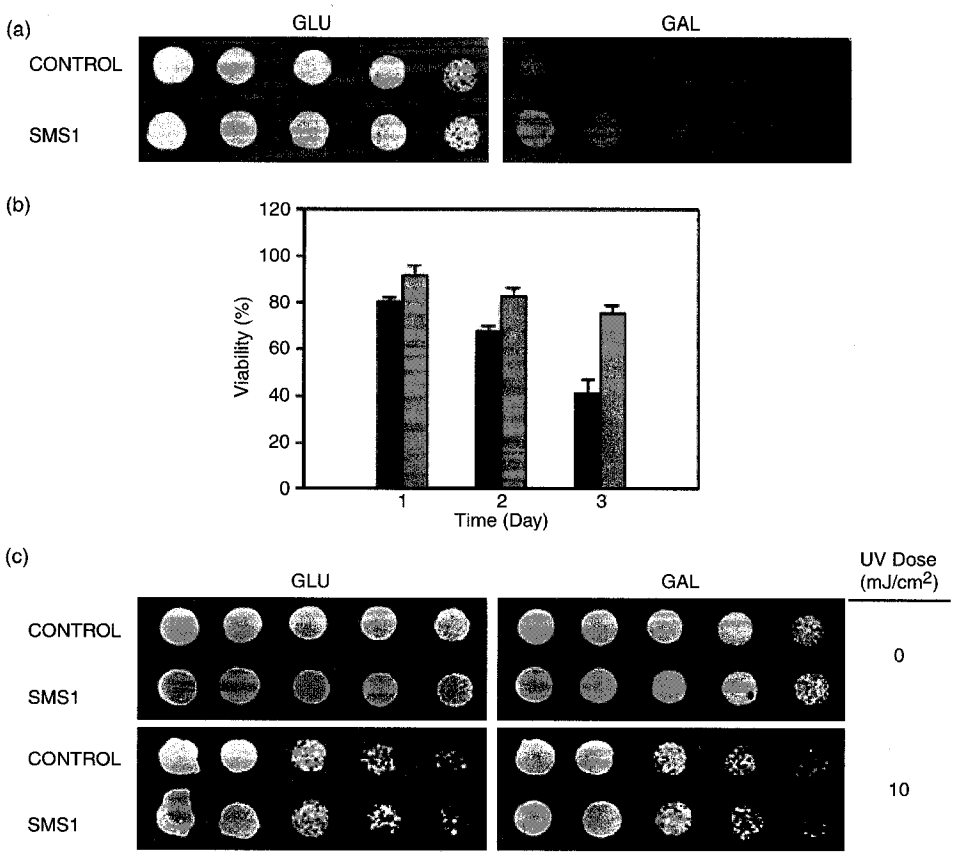

Fig. 4. The effect of high levels of salt and UV on the growth of SMS1-expressing yeast cells. (a) Freshly saturated glucose-grown cells were serially diluted and 10 μL aliquots were spotted onto glucose (GLU) and galactose (GAL) nutrient agar media containing 6% NaCl. A photograph of the plates after incubation at 30 °C is shown; (b) cultures of freshly saturated control (black) or SMS1 (gray)-expressing yeast transformants were used to inoculate fresh galactose media containing 6% NaCl. Aliquots of cells were taken at 24 h intervals and viability was determined by microscopical analysis of trypan blue-stained cells. The data are expressed as the mean percentage ± SD of viable cells and are typical of two identical experiments. (c) Aliquots of serially diluted control and SMS1-containing cells were spotted onto nutrient agar GLU- and GAL-containing media. The control plates received no further treatment, while the other plates were illuminated with 10 mJ cm^{-2} of ultraviolet light. The cells were allowed to grow at 30 °C and photographs of the plates are shown.

sphingomyelin (SM) and diacylglycerol (DAG) (Hannun & Luberto, 2004; Huitema *et al.*, 2004). Ceramide is an important sphingolipid that has been widely reported to be proapoptotic in mammalian cells and to inhibit yeast cell growth (Fishbein *et al.*, 1993; Nickels & Broach, 1996; Pettus *et al.*, 2002). In mammalian cells, the activation of SMS has been shown to prevent apoptosis (Taguchi *et al.*, 2004). The antiapoptotic properties of SMS are likely due to its ability to utilize ceramide as a substrate. An increase in the production of ROS leading to cellular damage such as lipid peroxidation has been implicated as being at least partly responsible for yeast cell death in response to stressful stimuli as diverse as Bax expression and H_2O_2, as well as high osmolarity (Madeo *et al.*, 1999; Huh *et al.*, 2002). Since SMS1 is capable of preventing cell death in response to these stresses, it remains possible that it also functions to promote survival by reducing ROS levels in yeast. Given its known function in mammalian cells, the ability of overexpressed SMS1 to utilize ceramide is nevertheless the most likely explanation for the observed anti-apoptotic properties of SMS1 in yeast. If this is true, it therefore makes sense that yeast cells expressing SMS1 would show an enhanced resistance to the cytotoxic effects of exogenously supplied ceramide or sphingolipids that can be converted to ceramide. To test this possibility we examined the ability of yeast cells expressing SMS1 to grow in the presence of exogenously supplied cell-permeable C_2-ceramide. Freshly saturated glucose-grown yeast cultures were used to inoculate galactose-containing media at low cell density. The cultures were grown overnight in the absence or in the presence of increasing concentrations of C_2-ceramide. As previously reported (Fishbein *et al.*, 1993), ceramide caused a dose-dependent inhibition of the growth of control cells (Fig. 5a). Although ceramide also inhibited the growth of

SMS1-expressing cells, the effect was more pronounced in control cells. In the presence of 2.5 μM ceramide, control cells grew only 35% as much as the untreated cells. In contrast, cells expressing SMS1 grew 76% as much as the untreated cells in the presence of 2.5 μM ceramide. SMS1-expressing cells also showed more growth than control cells in the presence of both 5 and 20 μM ceramide. The effect was less pronounced at 20 μM ceramide, possibly indicating that there is a limit to the amount of ceramide that can be enzymatically converted by SMS1. Nevertheless, these results suggest that SMS1 can protect cells from the growth-inhibitory effects of exogenous ceramide.

In yeast, phytosphingosine (PHS) is a sphingolipid intermediate that is converted to phytoceramide by the action of phytoceramide synthase (Dickson & Lester, 2002; Hannun & Luberto, 2004). Phytoceramide is then converted to inositol phosphorylceramide (IPC) by the action of the *AUR1*-encoded IPC synthase. Yeast cells lacking *AUR1* are not viable and accumulate high levels of phytoceramide when given PHS (Nagiec *et al.*, 1997). In addition, exogenously supplied PHS is cytotoxic (Skrzypek *et al.*, 1998). This suggests that Aur1p may function to rid the cell of unwanted and cytotoxic ceramides (Nagiec *et al.*, 1997). Given that the biochemical function of SMS1 is analogous to that of Aur1p

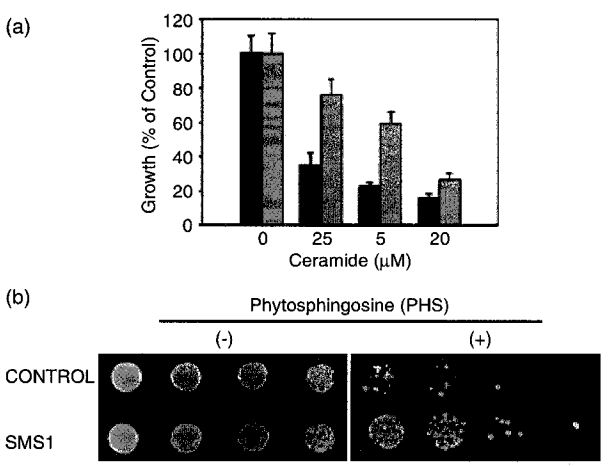

Fig. 5. SMS1 reverses the growth-inhibitory effects of exogenously supplied sphingolipids in yeast. (a) Cultures of freshly saturated cultures of control yeast cells (black) or SMS1 (gray)-expressing yeast cells were used to inoculate fresh galactose-containing YNB media at low cell density. The cultures received no addition (0) or increasing amounts of C_2-ceramide (2.5, 5 or 20 μM). The cultures were grown for 18–20 h at 30 °C and the turbidity of the cultures was determined at 660 nm. Growth of the different cultures is shown as a percentage of the growth seen in the control cultures. The data are mean ± SD of triplicate cultures and are typical of identical experiments. (b) Freshly saturated glucose-grown cells were serially diluted and spotted onto galactose-containing nutrient agar media with 0.05% Nonidet P-40 in the presence and absence of 20 μM phytosphingosine. The cells were allowed to grow for 4–5 days and a photograph of the resultant plates is shown.

in that both enzymes can use ceramide as a substrate (Obeid *et al.*, 2002), we tested the possibility that SMS1 would prevent the growth-inhibitory effects of exogenously supplied PHS. Glucose-grown yeast cells were serially diluted and spotted onto galactose-containing nutrient agar plates with or without 20 μg mL^{-1} PHS. Both the control and SMS1-containing yeast cells showed equal growth on plates containing no PHS (Fig. 5b). In contrast, yeast cells expressing SMS1 showed significantly more growth than control cells when grown in the presence of PHS. These results suggest that SMS1 is capable of promoting growth and preventing cell death in response to exogenously supplied sphingolipids that can be converted to phytoceramide. These results support the notion that SMS1 is anti-apoptotic by preventing stress-mediated increases in the levels of cytotoxic sphingolipids.

SMS1-expressing cells show enhanced resistance to chronic heat stress

In mammalian cells, an increase in ceramide occurs in response to numerous proapoptotic stimuli including heat shock (Jenkins, 2003). Sphingolipid metabolism as well as the enzymes involved in their synthesis and degradation are also important for heat shock responses in yeast (Dickson *et al.*, 1997; Wells *et al.*, 1998; Jenkins, 2003). The critical role of sphingolipids is demonstrated by the observation that yeast mutants that are unable to synthesize sphingolipids are unable to grow at elevated temperatures (Patton *et al.*, 1992). In the early stages of heat shock or stress, the levels of complex sphingolipids such as dihydrosphingosine (DHS) and PHS are transiently elevated. More prolonged heat stress eventually leads to the accumulation of different sphingolipids, including ceramide, the roles of which are largely unknown (Jenkins, 2003). Our results indicate that SMS1 can protect yeast cells from the growth-inhibitory and apoptotic effects of chronic stresses. Given that the only known function of SMS1 is to synthesize sphingomyelin and DAG using ceramide as a substrate, these results suggest that SMS1 protects yeast cells by decreasing stress-mediated increases in ceramide levels. Although stress-mediated increases in ceramide leading to growth inhibition and cell death have been observed in mammalian cells, it is not commonly reported in yeast (Jenkins, 2003; Riezman, 2004). The observation that SMS1 prevents the cytotoxic effects of exogenously supplied ceramide and PHS in yeast supports the idea that increased ceramide levels occur in response to multiple stresses in yeast (Fig. 5). Given that the long-term role of the observed elevation of sphingolipids and ceramide in response to heat stress is largely unknown, we used a spot assay to examine the ability of control and SMS1-expressing yeast cells to grow at different temperatures. Freshly saturated glucose-grown cultures of cells harboring the control

plasmid as well as pYES-SMS1 were serially diluted and plated onto nutrient agar media containing either glucose or galactose. The plates were subsequently incubated at 30, 37 or 41 °C for 3 days. Compared to cells grown at 30 °C, the control cells showed a reduction in growth at 37 °C which was more pronounced at 41 °C (Fig. 6). In contrast, SMS1-expressing cells showed more growth than control cells at 37 °C, and this effect was even more pronounced when cells were grown at 41 °C. These results demonstrate that SMS1 promotes the growth of cells in response to the chronic stress resulting from elevated temperature. This suggests that yeast cells, like mammalian cells, also respond to chronic heat stress by increasing the levels of ceramide.

Overexpression of the yeast AUR1-C also prevents the effects of high osmolarity

The yeast Aur1p is an inositol phosphorylceramide synthase that has similar biochemical properties as the mammalian SMS1 and possibly functional similarities (Obeid *et al.*, 2002). If SMS1 prevents stress-induced cell death in yeast, we reasoned that *AUR1* should also do the same. In order to analyze this possible role for *AUR1* in yeast, we cloned the aureobasidin A (AbA)-resistant *AUR1*-C *AUR1* mutant as a GFP fusion under the control of the galactose-inducible *GAL1* promoter into p425GAL1-GFP. Spot analysis of yeast

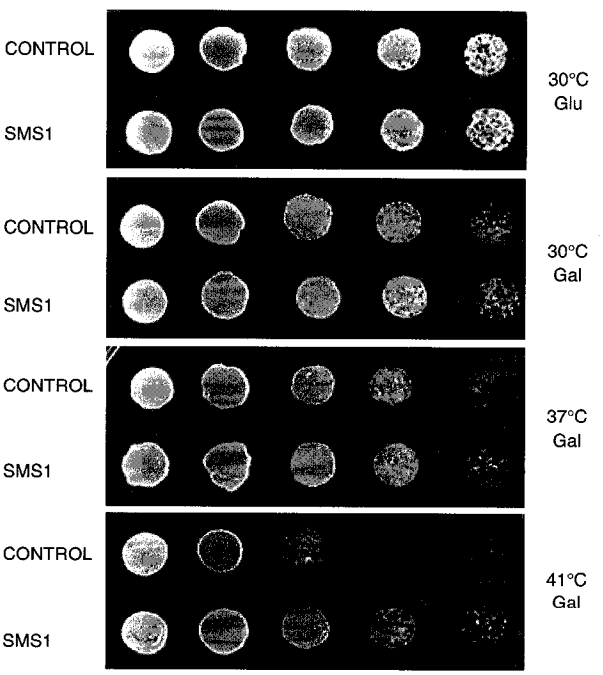

Fig. 6. The effect of temperature on the growth of SMS1-expressing cells. Freshly saturated glucose-grown cells were serially diluted and 10 μL aliquots were spotted onto glucose (GLU)- and galactose (GAL)-containing nutrient agar and incubated for 4–6 days at the indicated temperatures. Photographs of the resultant plates are shown.

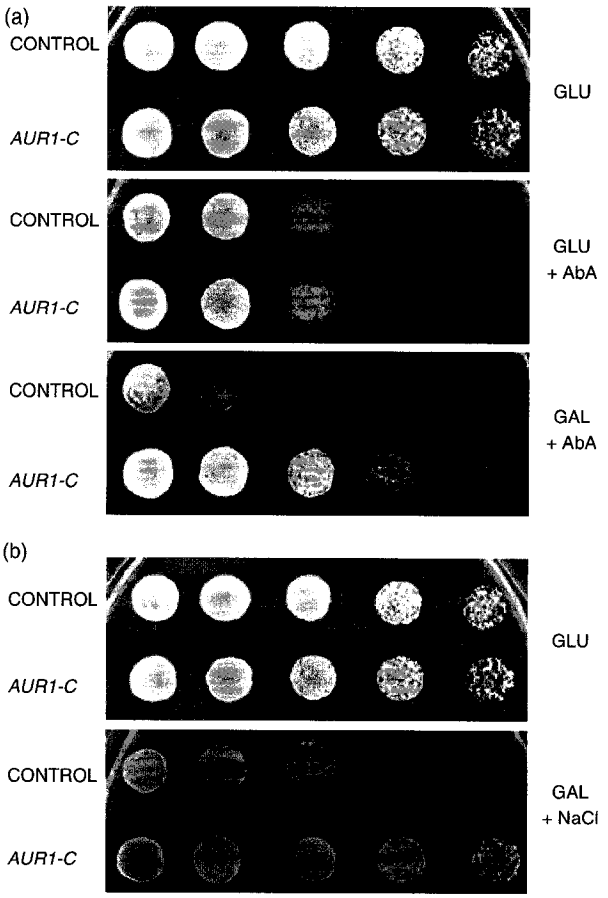

Fig. 7. The yeast *AUR1*-C protects against high-osmolarity-induced growth inhibition. The growth of yeast cells harboring the GFP-expressing plasmid p425ATG-GFP (CONTROL) or the AUR1-C-GFP-expressing plasmid (*AUR1*-C) was analyzed using the spot growth assay. (a) Cells were plated on YNB selective media containing glucose (GLU), GLU and 0.075 μg mL^{-1} of Aureobasidin A (GLU+AbA), or galactose and Aureobasidin A (GAL+AbA); (b) yeast cells harboring either the control or the *AUR1*-C-GFP-expressing plasmids were spotted on GLU-containing media or GAL media containing 1 M NaCl (GAL+NaCl).

cells harboring plasmid p425GAL1-*AUR1*-C-GFP on YNB selective media containing glucose, glucose and AbA, or galactose and AbA, suggests that the galactose-mediated expression of *AUR1*-C-GFP leads to AbA resistance in yeast (Fig. 7a). Yeast cells harboring either the control GFP-expressing plasmid p425GAL1-ATG-GFP or the *AUR1*-C-GFP-expressing plasmid were grown on glucose- or galactose-containing media with 1 M NaCl. Cells expressing *AUR1*-C-GFP showed enhanced growth in the presence of high levels of salt when compared to control cells (Fig. 7b). The viability of yeast cells grown in high levels of salt for 24 h was also enhanced by the expression of *AUR1*-C-GFP ($90.3 \pm 1.6\%$ for *AUR1*-C-GFP expressing cells vs. $81.2 \pm 1.3\%$, $n = 3$, for cells expressing only GFP). Although the effect was not as pronounced as with SMS1, the

expression of *AUR1*-C-GFP also increased the ability of yeast cells to grow at elevated temperatures (not shown).

In summary, we have identified mouse SMS1 as a suppressor of both the growth-inhibitory and cell death-inducing effects of a mammalian Bax in yeast. In addition, SMS1 was found to prevent the inhibitory effects of other stresses, including hydrogen peroxide, high levels of salt and elevated temperature. Consistent with its known biochemical function, SMS1 was also found to confer growth resistance to exogenously supplied ceramide or to the ceramide precursor phytosphingosine. The antiapoptotic role described for SMS1 in yeast suggests that this enzyme is at least in part responsible for the known anti-apoptotic role of SMS in mammalian cells. The enhanced ability of yeast cells that overexpress the *AUR1* gene to grow and survive in media containing high levels of salt also supports the role of inositol phosphorylceramide synthase as being anti-apoptotic. These results emphasize the usefulness of yeast as a model system to study the involvement of sphingolipids in the process of programmed cell death (Obeid *et al.*, 2002; Hannun & Luberto, 2004).

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control of the *GAL1* promoter show a galactose dependant inhibition of growth (Li et al., 2004). In an attempt to identify potential RGS1 interacting proteins, we have used these cells to screen a mouse T cell cDNA yeast expression library for sequences that will promote the growth of *RGS1* expressing cells (Yang et al., submitted for publication). In this way, we have cloned a 2364-bp cDNA encoding a 413-residue protein that is 97% identical to the human SMS1. We found that expression of *SMS1* in yeast not only suppresses the effects of *RGS1* overexpression, but also blocks the deleterious effects of a number of other stresses including the heterologous expression of mouse Bax, treatment with hydrogen peroxide, osmotic stress and elevated temperature. The anti-apoptotic effects of SMS1 in yeast are likely due to its biochemical property converting stress-mediated accumulation of ceramide to sphingomyelin.

Although *SMS1* cDNA sequences have been in the GenBank database prior to the identification of its function, the 5′ end of the *SMS1* cDNA that we isolated in our previous study represented a novel cDNA that contains a novel 5′ *UTR* sequence. This suggested that *SMS1* might be an alternatively spliced gene, giving rise to multiple transcripts. Here, we have analyzed the available *SMS1* cDNA sequences and the corresponding mouse genomic sequences in order to determine the structure of the *SMS1* gene. Our results indicate that the *SMS1* gene consists of at least 16 different exons that are alternatively spliced to generate four different transcripts and three different proteins. The regulation and possible functions of the different splice variants and proteins were also analyzed.

2. Materials and methods

2.1. Isolation of total RNA

Two-month-old Balb/c mice (Charles River Laboratories) were sacrificed by sodium pentobarbital overdose (150 mg/kg). The animals were surgically dissected, the tissues removed, washed in 1× phosphate-buffered saline (PBS), frozen in liquid nitrogen and stored at −80 °C. C2C12 cells were washed 3 times with ice cold PBS, collected by centrifugation and stored at −80 °C. RNA was extracted from tissues and cells using TRIzol essentially as described by the supplier (Invitrogen). The RNA was used for RLM–RACE and for RT–PCR analysis (see Sections 2.2 and 2.3).

2.2. RLM–RACE

RLM–RACE was used to amplify cDNA containing the entire 5′ or 3′ ends of the different *SMS1* transcripts essentially as described by the manufacturer (Ambion). For 5′RACE, total RNA extracted from different tissues (skeletal muscle for *SMS1*α1, kidney for *SMS1*α2 and testes for *SMS1*β and *SMS1*γ transcripts) was treated with calf intestinal phosphatase (CIP) for 1 h at 50 °C in order to remove 5′ PO_4 from degraded mRNAs and other RNAs. The RNA was then treated with tobacco acid pyrophosphatase (TAP) for 1 h at 37 °C. This removes the cap, thereby allowing the ligation of a specific adapter to the 5′ end of mature mRNAs. The RNA was then reverse transcribed into cDNA using M-MLV reverse transcriptase for 1h at 42 °C. The mRNA corresponding to the different SMS1 transcripts were then amplified using two separate PCR reactions. The first PCR reaction used an outer forward oligo that was specific for the RNA adapter (5′-GCTGATGGCGATGAATGAACACTG-3′) and a reverse outer oligo corresponding to a specific sequence present in the different SMS1 transcripts. The PCR products were then re-amplified using an inner forward oligo that was specific for the RNA adapter (5′-CGCGGATCCGAACACTG-CGTTTGCTGGCTTTGATG-3′) and a reverse inner oligo corresponding to a specific sequence present in the different *SMS1* sequences. The *SMS1* specific oligos were as follows: for *SMS1*α1, outer 5′-TGTGCTCCAAATGTACCCTGC-3′ (from exon 4) and inner 5′-CCGCCGGAATTCTTTCCACGC-3′ (from exon 2); for *SMS1*α2, outer 5′-ACTGTTCGTACAGG-GCAGGAC-3′ (from exon 9) and inner 5′-CCAAAGG-CAGCCTCCTGGAAA-3′ (from exon 8); for *SMS1*β, outer 5′-GAATTCTTTCCACGCGCCGAG-3′ (from exon 2) and inner 5′-ATG-CAGTCCGCCAGTCTACAG-3′ (from exon 1); and for *SMS1*γ, outer 5′-ACTGTTCGTACAGGGCAGGAC-3′ (from exon 9) and inner 5′-TATCAAGGCATGGCCCTGCAG-3′ (from exon 5). RLM–RACE was also used to obtain the 3′ end of *SMS1*β. In this assay, total RNA from testes was reverse transcribed into cDNA using an oligo dT containing adapter sequence. The cDNA was amplified by two separate PCR reactions. The first PCR used an outer reverse oligo that was specific for the oligo dT containing primer (5′-GCG-AGCACAGAATTAATACGACT-3′) and a forward outer oligo corresponding to a specific *SMS1*β outer sequence, 5′ GATGGGGACACTGAGTTTCTC 3′ (from exon 10). The PCR product was re-amplified using an inner reverse oligo that was specific for the oligo dT containing primer (5′-CGCGGA-TCCGAATTAATACGACTCACTATAGG-3′) and a forward outer oligo corresponding to a specific *SMS1*β sequence 5′-AGCCACGGGTTTACTTGGATC-3′ (from exon 10). PCR was performed at 94 °C for 45 sec, 60 °C for 30 sec and 72 °C for 45 sec (PCR with outer oligos) or 15 (PCR with inner oligos) for a total of 35 cycles followed by a final extension at 72 °C for 30 min. Aliquots of the PCR reactions were first visualized by ethidium bromide agarose gel electrophoresis and subsequently cloned into pCR2.1 (Invitrogen). Plasmid DNA containing 5 independently isolated PCR fragments for each *SMS1* transcript was sequenced.

2.3. Reverse transcriptase polymerase chain reaction (RT–PCR)

1 μg of total RNA was reverse transcribed and amplified by PCR using the ThermoScript RT–PCR system (Invitrogen). A reverse oligo 5′-CTGCCCATTGTCAGAGGAGAC-3′, corresponding to a common sequence in exon 9 was used to amplify all four *SMS1* transcripts (see Fig. 1 for location of oligos in the different transcripts). Sequence specific forward oligos as well as specific amplification conditions used for the different *SMS1* transcripts, were as follows: for *SMS1*α1 94 °C for 45 sec, 55 °C for 30 sec, 72 °C for 45 sec for a total of 31 cycles using the forward oligo: 5′-GGAAAGAATTCCGG-CGGCTGC-3′; for *SMS1*α2, 94 °C for 45 sec, 55 °C for 30 sec,

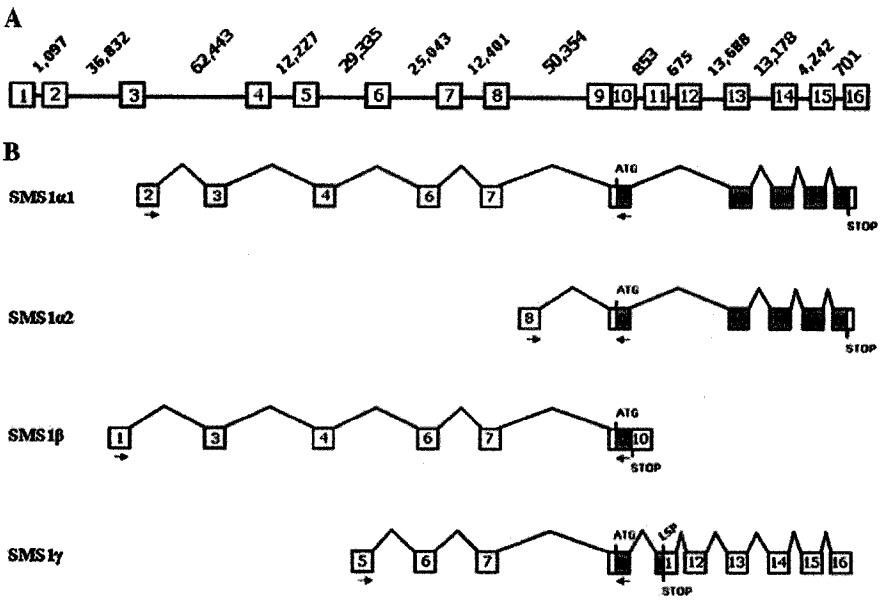


Fig. 1. Schematic representation of the genomic organization of the mouse *SMS1* gene. The sequence of the different mouse *SMS1* cDNAs was used to identify the *SMS1* gene in the mouse genomic DNA database. (A) The *SMS1* gene is located on a 265-kb fragment of DNA on chromosome 19. Exons are shown as numbered boxes. The sizes of the intervening (introns) are represented by lines and their lengths are shown in base pairs (bp). There is no intron between exons 9 and 10. (B) The exon composition of the different *SMS1* cDNAs is shown. *SMS1*α refers to the cDNA that we previously cloned while the other cDNAs were named *SMS1*α2, *SMS1*β and *SMS1*γ to reflect the different exons used and the different proteins. The location of the putative "ATG" that is used as the translational initiation site for all 3 forms of the SMS1 protein is shown in exon 9. Shaded areas within the different exons represent regions that are used as coding sequence. Arrows denote the location of primers used to amplify the different mRNAs by RT–PCR (see Fig. 4).

72 °C for 45 sec for a total of 35 cycles using the forward oligo 5′-TGTGCTTCCCAGAGGCGGCCT-3′; for *SMS1*β, 94 °C for 45 sec, 57 °C for 30 sec, 72 °C for 45 sec for a total of 32 cycles using the forward oligo 5′-TGTACCCCCGCTGTAGACTGG-3′; for *SMS1*γ, 94 °C for 45 sec, 57 °C for 30 sec, 72 °C for 45 sec for a total of 30 cycles using the forward oligo 5′-ACAGAGTGCAGAGGAGTCAGC-3′. As a control, β-*ACTIN* was amplified using these conditions: 94 °C for 45 sec, 50 °C for 1 min and 72 °C for 45 sec for a total of 25 cycles and using the forward 5′-GTGGGCCGCCCTAGGCACCAG-3′ and reverse 5′-CTCTTTGATGTCACGCACGATTTC-3′oligos. Transcript for the second mammalian SMS encoding gene, *SMS2*, was amplified as follows: 94 °C for 45 sec, 55 °C for 30 sec, 72 °C for 45 sec for a total of 30 cycles using the forward oligo: 5′-CCAACGGGTTACGAAAGG-3′; and reverse oligo: 5′-GCCGCTGAAGAGGAAGTC-3′. An aliquot of each PCR reaction was separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, visualized, and photographed under UV illumination.

2.4. Cell culture

The mouse skeletal muscle C2C12 cell line was used to test the effect of TNF-α on the expression of the different SMS1 transcripts. C2C12 cells were grown in DMEM supplemented with 10% Fetal Bovine Serum and 1% mixture of penicillin–streptomycin at 37 °C in 5% CO_2. Cells were grown to 70% confluency and TNF-α was added to the culture medium at a concentration of 50 ng/ml. Total RNA was extracted 24 h later. The RNA was used to determine the levels of the different SMS1 transcripts by RT–PCR as described in Section 2.3.

2.5. Yeast strains and plasmids

Strain BY4741 (*MAT***a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) was used as the wild type yeast cell. The yeast expression vectors to express SMS1α (pYES2-SMS1α), Bax (pGilda-Bax) as well as the corresponding empty control vectors (p425GAL1, p246GAL1 and the GFP expressing vector p425-ATG-GFP) were previously described (Kong et al., 2002; Somerville et al., 2003). The SMS1β–GFP expressing vector was constructed by amplifying the coding sequence of SMS1β by PCR using pYES2-SMS1α1 DNA as a template with the following forward 5′-CTAGTGGATCCCCCGGGCTGCAGGAATTC-GGCAAGCTGGGGGTACTGAATG-3′ and reverse 5′-CATG-GTGGCGATGGATCCCGGGCCCGCGGTACCGTTG-TATTTTAAGAGCAGCCA-3′ oligonucleotide primers essentially as previously described (Kong et al., 2002). The PCR product was ligated into the GFP vector in vivo by co-transforming the PCR product into yeast with *Hin*dIII linearized p426GAL1–GFP as previously described (Oldenburg et al., 1997).

2.6. Yeast growth and transformation

Synthetic minimal media consisting of yeast nitrogen base (YNB) containing 2% glucose supplemented with the appropriate amino acids and bases was routinely used to grow yeast (Kong et al., 2002; Somerville et al., 2003). Glucose was replaced with 2% galactose and 2% raffinose in order to induce

expression of sequences under the control of the *GAL1* promoter (Li et al., 2004). Plasmids were introduced into yeast using lithium acetate and the resultant transformants were selected and maintained by omitting the appropriate nutrient from the growth media. The plasmids used contained the following selectable markers: p425GAL1, *LEU2*; pGilda-Bax and p423GAL1, *HIS3*; and pYES2, *URA3*. To determine the effects of expressing *SMS1*β on the growth of *SMS1*α- and *BAX*-expressing cells, the spot growth assay was used essentially as described (Li et al., 2004). Briefly, freshly saturated glucose cultures of the different transformants were serially diluted (5 : 1) and 10 μl aliquots of each dilution was spotted on nutrient agar plates containing either glucose or galactose and subsequently incubated at 30 °C for 3 to 4 days.

2.7. Western blot analysis

Soluble yeast protein was extracted by heating NaOH treated yeast cells in SDS–PAGE loading buffer for 3 min at 95 °C (Jean-Baptiste et al., 2005). An equal amount of protein of the different samples was separated by SDS–PAGE, transferred to membrane and sequentially challenged with monoclonal anti-GFP antibody (Santa-Cruz Biotech) and with HRP-conjugated donkey anti-goat IgG 2° antibody. Signals were subsequently developed using chemiluminescent luminol reagent (ECL+) and exposure to X-ray film.

3. Results and discussion

3.1. Organization and alternative splicing of the SMS1 gene

In addition to the cDNA identified in our previous study (GenBank accession no.: AY509044), three other mouse *SMS1* encoding cDNAs that differ in their 5′ and 3′ non-coding sequences were found in the GenBank database (accession nos.: BC019443, AK082974 and AK076554). These differences suggested that the cDNAs represent mRNAs that originated from alternative splicing of a single gene. In order to characterize the observed differences as well as the structure of the *SMS1* gene, we used BLAST to compare the nucleotide sequences of the four *SMS1* cDNAs with the mouse genomic database. All *SMS1* cDNA sequences were located within 16 different exons that were spread over a 264 kb portion of mouse chromosome 19 (GenBank accession no.: NT_039687) (Fig. 1A). The four cDNAs show significant differences in the combinations of exons used (Fig. 1B). Analysis of the predicted open reading frames of the *SMS1* cDNAs suggested that there also exist three different SMS1 proteins. A nomenclature was adopted in order to differentiate between the different SMS1 transcripts and proteins (Figs. 1B and 2). The alternatively spliced *SMS1*α mRNAs, *SMS1*α1 and *SMS1*α2, refer to the two different transcripts that code for the previously characterized 413-residue SMS1α protein (Figs. 1B and 2). *SMS1*β and *SMS1*γ refer to mRNAs that encode C-terminally truncated

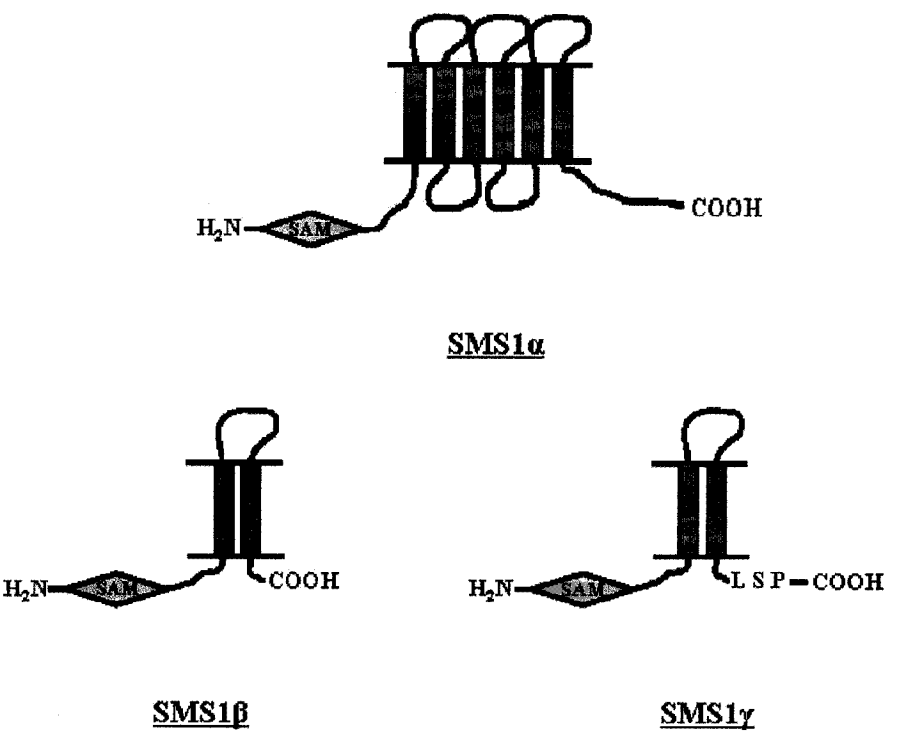
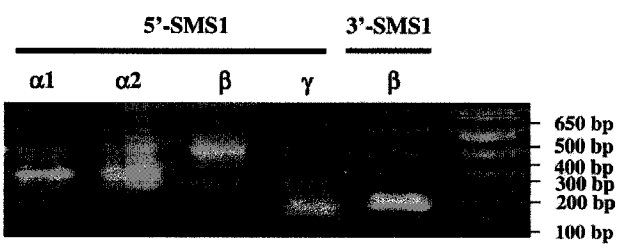


Fig. 2. Schematic representation of the SMS1 proteins. Alternative splicing of the *SMS1* gene gives rise to 4 different transcripts as well as 3 different proteins that we have named SMS1α, SMS1β and SMS1γ. SMS1α contains 413 residues and is predicted to be an integral membrane protein. The topology as well as the N-terminal sterile alpha motif (SAM) of SMS1α is shown. SMS1β and SMS1γ refer to C-terminally truncated forms of the SMS1α protein. SMS1β consists of the N-terminal 208 residues of SMS1α. SMS1γ is identical to SMS1β with the addition of the tripeptide LSP to its C-terminus. The N-terminal SAM domain as well as the two predicted N-terminal TMDs of SMS1β and SMS1γ are shown.

forms of the SMS1α protein. SMS1β consists of the N-terminal 208 residues of SMS1α. SMS1γ is identical to SMS1β except that there is an addition of the tripeptide LSP to its C-terminus. Both SMS1β and SMS1γ contain the N-terminal sterile alpha motif (SAM) domain as well as the first two of the six transmembrane domains (TMDs) found in SMS1α. A schematic diagram is shown in order to illustrate the structural differences between the different SMS1 proteins (Fig. 2).

Of the 16 *SMS1* exons, numbers 1 through 8 contain all the non-coding 5′ *UTR* sequences. Alternative splicing of these exons gives rise to different and unique 5′ *UTR*s for the 4 *SMS1* mRNAs (Fig. 1B). Exon 9, the only exon that is common and present in all 4 mRNAs, contains the translational start site, the SAM motif as well as the first two putative TMDs of SMS1. Exon 9 is spliced onto exons 13 through 16 to make the 3′ ends of both *SMS1*α1 and *SMS1*α2 transcripts. The 5′ *UTR* of *SMS1*β consists of exons 1, 3, 4, 6 and 7 while exon 9 is spliced onto exon 10 to make up the coding portion as well as the 3′ *UTR* of the transcript (Fig. 1B). The 5′ *UTR* of *SMS1*γ consists of exons 5, 6 and 7 while exon 9 is spliced onto exon 11 to make up the

A



B

Exon 8: 5' end of SMS1α2

CTACATTCCAAAAACCCTGTGCAGCCTGAGGCGCGCGGGCCGTGCTCCTGT*AGTCCCTTGAGCTCCGTGT
CCTCTGCGTCTGGCTGTGCTTCCCAGAGGCGGCCTGTGGGGACCGCTGGCCACTCCCAACCGCTGGCCAGC
TGCACGCAGAGACCCGCGTAAACTTGTCAGGAACTTTCCAGGAGGCTGCCTTTGGGAGGAGAG

Exon 1: 5' end of SMS1β

CCAGAGATTCCAGGACCCCAGGTGACACCATCACCATCCAAGCTAACCAGCAGCAGGAGAAAAGCATGGAG
GAGAGCGACGGCTGTCCAAACATCTTTCGCCACCTTCATCCTCCGTCGCCGGGAATGGGTCAGAACAGGGA
AGCAGCAGGAGGAGCGATGCCAGCGCCTTAGGAACGGGAACTCCTCTTCCCA*ACTTCCGCCCCTGGGGAA
AAGCAGAAGCCATCAGCTCCAAGAGAGCGGCTCTCCAATTCTTAGCCGTTAGGTTCTGGCTCTGGAGACAG
CCGGTCCTCCCGGTGTCACCGGCGTCGCAGTACCCTCGCTGTACCCCCGCTGTAGACTGGCGGACTGCATG
CAG

Exon 5: 5' end of SMS1γ

CAGGGAA*GGGCAGCTTGAGGTCACCACAGAGTGCAGAGGAGTCAGCGTCCTGCAGGGCCATGCCTTGATA
TGTCG

Exon 10: 3' end of SMS1β

GTAAGTAGAGTAAACACTCTTGAGATCCAGCCATGAAGGGGTTTGCACACAGGGATCCAGTTATTGGAAAT
GAAATTTACCAAGTGTTGAAATCAAAGCTTAGGAATTCCCTTTCCTTTCCATTCTGAAGCCACACACCCAT
GGAACTAATTGAAGCTCCTACAGCTTTGAGAGGATGATGTATTTGTTGCCTGTTCGCTACAAGATTGCTCC
AGAATTAAGACCATGAGCAATGCTAGTCTATTTTCAAGCAGTAGGAAAGAAAACACTTAGACATCTTCAGA
GAGCTGAGGCTGTTGAGTAACAGAGGGGGAAACACCTACCTAGTCTTGAATGGTGTTTACTCAGGTGAATT
GTTGCCAACCTCCCACCCCTGTCAGACCTAGAACCTCATCACTAAGTATTCACAGTTTTTGACATGTTTTA
GACAGATATTCTACAATGACTTTGCTTGGGAAAAATTTGATTATCTCATTTAATTTAAGCAGGAAGCATGG
TGCAGAATCTATTTGATTCCATCTTACAGGATGTGTGGATAAGTACCTGTATTCACTTTTAGTTGGTTCCA
CTGTGATTTTCACGTCCCAAGCTCTAAACACAACGGGGACCGCCCAAAGGGAGCTGTTTACTTAGGAACAC
CTCGTCCCTTCCTTTAATTAACTGCGCTTGTTCATGCTGTCTGGTTGTCTTCCGAGTGGACCACCTTGTGG
CATTCCTGGATGGTGTTGGGAATTACAGTTTCTCCCTACCCACTGGATGTAGGAGCATCAGTAGGAGGTAC
TAGCCACGGCCCAAGCTTGCTGCTCTTGTGCCTTGGACAGTGTCACCAAATCCACAGAGTGAAAAAGGCTA
TCCCTGGGACTGAGGCACTCATCCCTGGAACTGCCACTCTCTGTGTCACTCTGTGTGTCTGCTACTGATGA
TGGGGACACTGAGTTTCTCTGTAGTAGACTGAGTTGCCTGAGCCCAAGTATGGCTGGCTATTAAAAGGAGT
CATATTCCATCAAGAAAACAAAATGCAGGTGGAGTGTTCCTGTTGAACTTTTCTGAGTGGTTTCCTGGTGT
TGGATAAAGTCTCTCAGCTTATGAAGTCATGTGTCTTCAAGCAGAAGGAGTAGGCCCCTGCTGGCTTTGCC
AGGGGACCCTCGTCCCTGTGAGAAGTCACTGACATAGCAGCCACGGGTTTACTTGGATCTAGCTTGACAAT
TTTATAAGTGAAGTAGAAGTTTGAATAGAATTGATGGGCTATGCTACCGTGGAGGTGTTGG*AGAAAAAAA
AGGAAAAAAAAA

Fig. 3. RLM–RACE amplification and analysis of the 5′ and 3′ ends of the *SMS1* transcripts. For 5′ RACE, an RNA adapter was ligated onto the 5′ end of total RNA. The RNA was then made into cDNA using reverse transcriptase. For 3′ end RACE, the RNA was made into cDNA using reverse transcriptase and an oligo dT containing adapter sequence. The RNA was then amplified by two separate PCR reactions using both outer and inner oligos corresponding to the RNA adapter or the oligo dT containing adapter sequence and to the different *SMS1* transcripts. (A) An aliquot of the final PCR products of 5′ RACE (*SMS1*α1, *SMS1*α2, *SMS*1β and *SMS1*γ) and 3′ RACE (*SMS*1β) reactions were separated by agarose gel electrophoresis and a photograph of the ethidium bromide-stained gel is shown. The sizes of the molecular weight markers used are shown on the left of the gel. (B) An aliquot of the PCR products was ligated into pCR2.1 and the nucleotide sequence of five plasmids containing the different insert was determined. The nucleotide sequences were compared to the corresponding GenBank cDNA sequences and the appropriate mouse genomic sequence. The complete sequence of the exons containing the 5′ ends and the 3′ end of the *SMS1* transcripts analyzed are shown. The underlined sequence corresponds to new sequences determined by RLM–RACE and corresponds to an extension of the sequences present on the cDNA sequences.

coding portion and 3′ *UTR* of the transcript. Exons 12 through 16 make up the 3′ *UTR* of the *SMS1*γ transcript (Fig. 1B).

3.2. Mapping the 5′ and 3′ ends of the SMS1 transcripts

Although the 5′ and 3′ ends of most *SMS1* exons were readily identified by comparing the sequences of the cDNAs and genomic DNA, the 5′ ends of the different transcripts are not available by using such an analysis. This is because the 5′ ends of the different *SMS1* transcripts are encoded by unique exons. For example, exon 2 contains the 5′ end of *SMS1*α1, exon 8 contains the 5′ end of *SMS1*α2, exon 1 contains the 5′ end of *SMS1*β and exon 5 contains the 5′ end of *SMS1*γ (Fig. 1B). We therefore used a modified 5′RACE, RLM–RACE, to identify the 5′ ends the *SMS1* transcripts (Alvarez et al., 2005; Ramanathan et al., 2005). This technique involves the ligation of a specific linker onto the 5′ ends of mature transcripts, which then permits the selective amplification of capped mRNA. Using a 2-step amplification process with different combinations of oligos corresponding to outer and inner segments of the ligated linker as wells as outer and inner oligos corresponding to the desired transcript, we amplified PCR product corresponding to the 5′ ends of all four alternatively spliced *SMS1* mRNAs. An aliquot of each PCR reaction was separated by agarose gel electrophoresis (Fig. 3A). Single bands of 300, 300, 400 and 100 bp corresponding to *SMS1*α1, *SMS1*α2, *SMS1*β and *SMS1*γ transcripts were respectively amplified. The PCR products were subcloned and the nucleotide sequence determined from five different clones for each transcript. The deduced nucleotide sequences were compared to the sequences of the 5′ ends of the *SMS1* cDNAs as well as to the nucleotide sequences of the corresponding region of the mouse genomic DNA (Fig. 3B). The available sequences of the 5′ ends of the different transcripts were extended by the following: 51 nt for *SMS1*α2 (GenBank accession no.: BC019443), 194 nt for *SMS1*β (GenBank accession no.: AK082974) and 7 nt for *SMS1*γ (GenBank accession no.: AK076554). The nucleotide sequence of the RACE PCR products suggested that the 5 'end of the *SMS1*α1 cDNA (GenBank accession no.: AY509044) corresponds to the 5′ end of the transcript.

Exon 16 contains the 3′ end of 3 of the 4 *SMS1* transcripts including *SMS1*α1, *SMS1*α2 and *SMS1*γ (Fig. 1B). The nucleotide sequence of the *SMS1*α1 cDNA includes a poly A tail that is not present in the corresponding genomic sequence. In addition, a consensus polyadenylation sequence (AATAAA) is located 19 nts upstream of the 3′ poly A tract (Zhao et al., 1999). This indicates that we have identified the 3′ end of the transcripts containing exon 16 at their 3′ ends. The 3′ end of *SMS1*β is unique since it consists of exon 10 (Fig. 1B). There is no poly A tail present at the 3′ end of the *SMS1*β cDNA sequence. We therefore used RLM–RACE to amplify, clone and determine the 3′ end of the *SMS1*β transcript (Fig. 3A). Sequence analysis of the cloned 3′RACE *SMS1*β PCR product allowed us to extend the 3′end of the cDNA sequence by 21 nt (Fig. 3B). Although this sequence has a poly A sequence at its 3′ end, this sequence is present in the genomic DNA (Fig. 3B). In addition there is no consensus polyadenylation sequence within the 500 nt either 5′ or 3′ of the end of the *SMS1*β sequence. Although the *SMS1*β 3′ RLM–RACE PCR product clearly consists of a single band when visualized on an ethidium bromide stained agarose gel (Fig. 3A), this band apparently does not represent the 3′ end of *SMS1*β. We cannot explain the absence of a poly A tail at the 3′ end of the amplified *SMS1*β transcript.

The sequences of the intron/exon boundaries as well as the sizes of the different exons were determined by comparing the sequences of the *SMS1* transcripts and the corresponding genomic DNA (Table 1). With the exception of the boundary between exons 9 and 10, all the exon/intron boundaries follow

Table 1
Sequences of the exon–intron junctions of the alternatively spliced mouse *SMS1* gene

Exon[a]	Exon size (bp)	Splice acceptor site (intron–exon)[b]	Donor acceptor site (exon–intron)
1	357	5′ end*f*CCAGAGATTC	CTGCATGCAG–gtgagggcgg
2	244	5′ end*f*GAGGTTCCAA	TCGCGTGCAA–gtgagtcgcg
3	79	gtgcttgcag–GGTGAAGGCA	GACTCCGAAG–gtgagtagag
4	91	gttattctag–GGAAAGCTTC	GACAGCACAG–gtaagtgaaa
5	75	5′ end*f*CAGGGAAGGG	TGATATGTCG–gtatgggttt
6	43	tttctcctag–ACCGGAAGAA	CATGATTGAG–gtaaatggac
7	82	ccggtaacag–GTAATGGAGA	AAAATAATCC–gtaagtaaaa
8	204	5′ end*f*CTACATTCCA	GGGAGGAGAG–gtacgtgtgg
9	840	gctcccacag–AAGGAAGAAT	TAAAATACAA–gtaagtagag
10	1290	taaaatacaa–GTAAGTAGAG	AAAAAAAAAA*f*3′ end
11	57	ttggaagcag–ATTGTCGCCT	CCCAGTACTG–gtatgttgca
12	78	ccccatttag–AATTGGACTT	TGCAAACCAG–gtacccctg
13	118	ctttttctag–GTCTATTATT	TTCTCCGAAG–gtaaactact
14	154	tttctggtag–CTCTTTGGAG	ATCAAAGAGT–gtaagtctta
15	167	ctccttctag–ATTCTCCTCG	CAATCAGCAA–gtgagtcgcc
16	544	gctttcctag–GTGCTTAAGG	CTTGGGAAAA*f*3′ end (A_n)

The intron/exon boundaries were determined by comparing the sequences of all the *SMS1* cDNAs (see Fig. 1) with the mouse *SMS1* containing genomic DNA sequence found on chromosome 19 (GenBank accession no.: NT_039687). Introns are shown in lower case and exons in upper case letters.

[a] The 5′ end of exons 1, 2, 5 and 8 contain the respective 5′ ends of the transcript for *SMS1*β, *SMS1*α1, *SMS1*γ and *SMS1*α2. Exon 10 contains the 3′ end of *SMS1*β while exon 16 contains the 3′end of *SMS1*α1, *SMS1*α2 and *SMS1*γ. RLM–RACE PCR was used to determine the location of the 5′ ends of all 4 *SMS1* transcripts as well as the 3′ end of *SMS1*β. The 3′ end of exon 16 was identified by the presence of a poly A tail in the *SMS1*α1 cDNA used in this study.

[b] There is no intronic sequence between exons 9 and 10.

the GT/AG rule for splice sites. The exception reflects the fact that there is no intron between exons 9 and 10. Exon 10 is therefore both an exon as well as an intron. The splice sites of these coding introns may differ from the typical introns (Ast, 2005).

3.3. Tissue distribution of the SMS1 transcripts

In order to confirm that the cDNAs represent expressed transcripts, semi-quantitative RT–PCR analysis was performed using total RNA isolated from a panel of mouse tissues. PCR products corresponding to the predicted size were detected for all four cDNAs, suggesting that they represent expressed transcripts (Fig. 4). The *SMS1*α1 and *SMS1*β transcripts are widely distributed, the *SMS1*α2 transcript shows a more narrow distribution while the *SMS1*γ transcript is detected only in testes and heart. Taken together, these results suggest that the regulation and functions of the different SMS1 transcripts and proteins are likely to be tissue specific. Mammalian cells also contain a second SMS gene called *SMS2* (Huitema et al., 2004). Although SMS2 has SMS activity, it lacks the N-terminal SAM domain present in SMS1. We also examined the tissue distribution of *SMS2* transcript using RT–PCR. The *SMS2* transcript is widely distributed indicating that many cells are likely to express both *SMS1* and *SMS2* (Fig. 4).

3.4. Regulation of SMS1 gene expression in response to TNF-α

SMS catalyzes the production of sphingomyelin and diacylglycerol (DAG) using ceramide and phosphatidylcholine as substrates (Hannun et al., 2001; Hannun and Luberto, 2004). Activation of SMS prevents apoptosis by a mechanism that likely involves a decrease in the levels of the pro-apoptotic ceramide (Hannun et al., 2001; Taguchi et al., 2004). Regulation of SMS activity has been reported to occur in response to both mitogenic and apoptotic stimuli (Riboni et al., 2001; Itoh et al., 2003; Watanabe et al., 2004). Mammalian cells contain two SMS genes namely *SMS1* and *SMS2* (Hannun and Luberto, 2004; Huitema et al., 2004). Since the *SMS* genes have only recently been cloned (Huitema et al., 2004; Yamaoka et al., 2004), the regulation of either enzyme has not been fully addressed. As a first step towards examining the regulation of *SMS1*, we determined the levels of *SMS1* transcripts in cultured C2C12 cells stimulated with the pro-apoptotic cytokine TNF-α. Total RNA was extracted from untreated C2C12 cells as well as C2C12 cells treated with TNF-α for 24 h. The RNA corresponding to the different *SMS1* transcripts were amplified by RT–PCR using transcript specific oligos. Three of the four *SMS1* transcripts, α1, α2 and β, were detected in control C2C12 cells (Fig. 5A). A modest increase in all 3 transcripts is detected in cells treated with TNF-α. *SMS1*γ was not detected in either control or TNF-α treated cells. As a control, we also amplified the transcript corresponding to β-*ACTIN*, the levels of which were found to remain constant in control as well as TNF-α treated cells. As a control, we also show that TNF-α induced the cleavage of PARP and caspase 3, indicating that TNF-α is capable of initiating apoptosis in these cells (Fig. 5B). These results suggest that the *SMS1* gene may be transcriptionally regulated by pro-apoptotic stimuli like TNF-α. Given that SMS1 is likely to be anti-apoptotic, an up-regulation

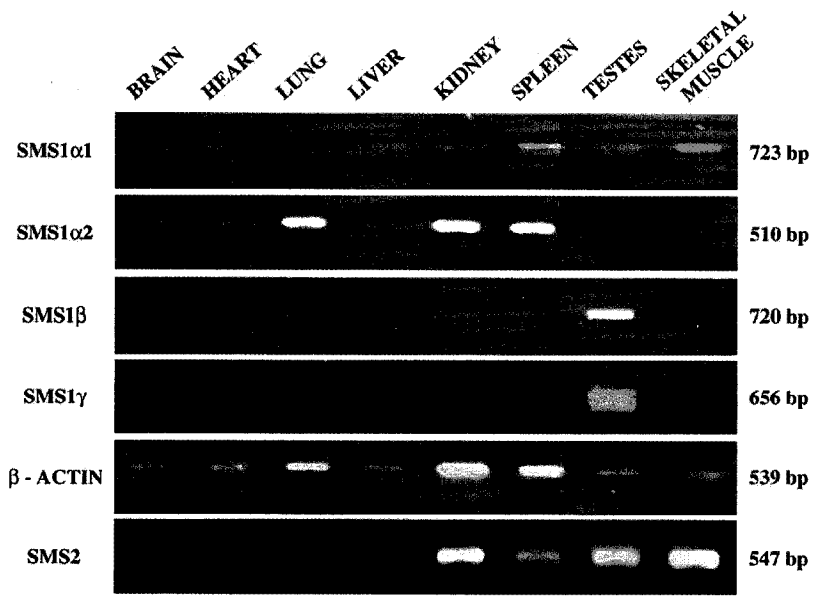


Fig. 4. Tissue distribution of the *SMS1* transcripts. Total RNA was isolated from a variety of mouse tissues, reverse transcribed and the different *SMS1* transcripts were amplified using the ThermoScript RT–PCR system (Invitrogen). A reverse oligo corresponding to a sequence in exon 9 that is present in all four *SMS1* transcripts as well as transcript specific oligos were used to amplify all four *SMS1* transcripts (see Fig. 1 for the locations of the oligos). As a control, β-*ACTIN* was also amplified. An aliquot of each PCR reaction was separated by agarose gel electrophoresis, stained with ethidium bromide and a composite of the resultant photographs are shown. The size in base pairs (bp), of each PCR product corresponding to the predicted size of the different *SMS1* transcripts is shown on the right. Similar results were obtained in two separate experiments.

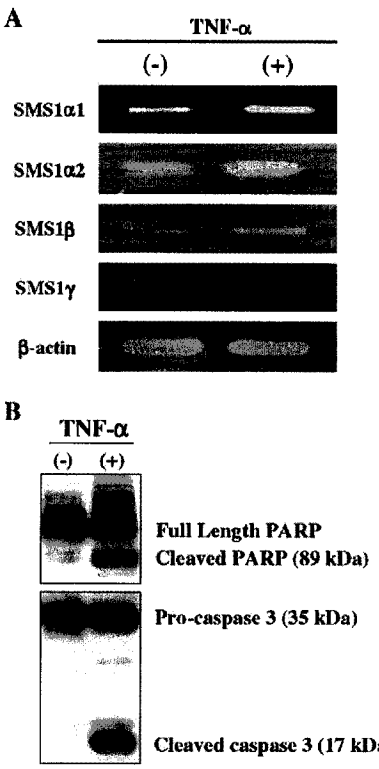


Fig. 5. Analysis of the effects of TNF-α on the expression of *SMS1* transcripts. (A) Total RNA was extracted from untreated (—) as well as TNF-α treated C2C12 cells. The mRNAs corresponding to the different *SMS1* transcripts were amplified by RT–PCR using transcript specific primers (see legend to Fig. 3). An aliquot of each PCR reaction was separated by agarose gel electrophoresis and a composite of the ethidium bromide-stained gels is shown. The levels of β-*ACTIN* were also determined and served as a loading control. (B) Soluble protein was extracted from untreated (—) as well as TNF-α treated C2C12 cells. The extracts were analyzed by Western blot using a monoclonal anti-PARP antibody (top panel) which recognize both the full length (inactive) and cleaved PARP (89 kDa) and a monoclonal caspase 3 antibody that recognizes both the pro-caspase (35 kDa) as well as the cleaved form of caspase 3 (17 kDa).

of the levels of *SMS1* transcripts may represent a response to stress that attempts to counteract the stress and in order to try avoiding cell death. Such an up-regulation in response to stress has been observed for a number of other anti-apoptotic genes in response to apoptotic stimuli (Ito et al., 2004; Coles et al., 2005). The regulation of *SMS1* gene in other cells and in other conditions remains to be determined. A previous study has demonstrated that an increase in the nuclear levels of ceramide is responsible for Fas-induced apoptosis in Jurkat T-cells (Watanabe et al., 2004). A decrease in nuclear, but not microsomal, SMS activity correlated with the stress mediated increase in ceramide. The loss of SMS activity was rapid since over half the SMS activity disappeared within 6 h of Fas-induced apoptosis. This indicates that post-translational mechanisms also serve to regulate the levels of SMS. Although the molecular mechanisms responsible is not known, it is interesting to note that preventing caspase 3 activation served to inhibit the loss of SMS activity. It remains to be determined the importance of this process in other cell types as well as the relative contribution of *SMS1* and *SMS2* to the process.

3.5. Analysis of SMS1β protein in yeast

The 413 residue SMS1α protein has been shown to possess sphingomyelin synthase activity when overexpressed in either mammalian or yeast cells (Huitema et al., 2004; Yamaoka et al., 2004). The function of the truncated SMS1 proteins encoded by the *SMS1*β and *SMS1*γ transcripts has not been investigated. The SMS1β and SMS1γ proteins contain the first two of the six putative TMDs found in SMS1α (Fig. 2) and are therefore unlikely to have sphingomyelin synthase activity (Huitema et al., 2004). Like SMS1α , these truncated proteins nevertheless contain the N-terminal SAM motif. The ca. 70 amino acid SAM domain has been shown to interact with SH2 or other SAM domains (Kim and Bowie, 2003). This motif which is present in a large number of different proteins, many of which are involved in signal transduction. For example, the yeast SAM containing Ste50 protein is involved in modulating a number of signaling responses including the ability to respond to mating pheromone. The ability of Ste50p to interact with the Ste11p kinase through their common SAM domain is necessary for its function (Ramezani-Rad, 2003). It is known that proteins containing SAM domains, such as SMS1, are capable of forming both homodimers as well as heterodimers (Kim and Bowie, 2003). Thus it is possible that SMS1β exerts its function by interacting with SMS1α. Such an interaction may lead to an increase or a decrease in SMS1α function. We have previously demonstrated that SMS1α inhibits apoptosis in response to a number of stimuli including the expression of murine Bax in yeast (Yang et al., submitted for publication). We therefore used the spot growth assay to examine the possibility that SMS1β may decrease or enhance the ability of SMS1α to prevent Bax-mediated cell death in yeast. Yeast cells were transformed with empty plasmids or with different combinations of *BAX*-, *SMS1*α- and *SMS1*β-expressing plasmids. The transformants were grown to saturation in glucose containing yeast media, serially diluted (fivefold), and 10 μl aliquots were spotted onto selective nutrient agar plates containing either glucose to repress the expression or galactose to induce the expression of *BAX*, *SMS1*α and/or *SMS1*β. The plates were then incubated at 30 °C and the cells allowed to grow for 3 to 4 days. No difference was observed in the growth of all the transformants on glucose-containing media (Fig. 6A, left panel). Similarly, no differences were observed between the growth of control, *SMS1*α or *SMS1*β-expressing cells on galactose media (Fig. 6A, right panel). In contrast, the growth of cells expressing Bax was inhibited on galactose media. As previously demonstrated (Yang et al., submitted for publication), co-expression of *SMS1*α largely abolished the growth inhibitory effects of Bax (Fig. 6A, right panel). In contrast, cells co-expressing *SMS1*β with *BAX* showed the same growth inhibitory effects as cells expressing Bax alone. Yeast cells co-expressing *SMS1*β along with *BAX* and *SMS1*α showed the same growth as cells co-expressing Bax and SMS1α. These results indicate that SMS1β is unable to prevent the growth inhibitory effects of Bax and nor does it interfere or enhance the effects of SMS1α on Bax.

As a control, we performed Western blot analysis on cells harboring the *SMS1*β construct. The *SMS1*β used in this study

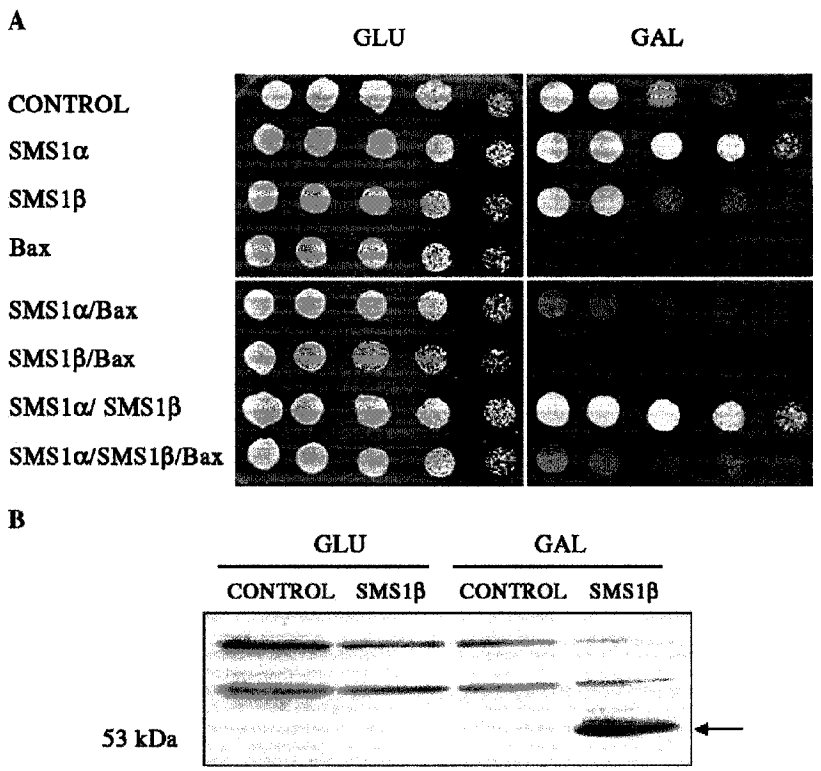


Fig. 6. Functional expression and analysis of SMS1β in yeast. (A) Yeast transformants that contain only empty p423GAL1, p425GAL1ATG–GFP and pYES2 vectors (control) were compared to cells harboring either plasmid p423GAL1, p425GAL1ATG–GFP and pYES2–SMS1 (SMS1α); p423GAL1, pYES2 and p425GAL1–SMS1 β-GFP (SMS1β); pGilda-Bax, pYES2 and p425GAL1ATG–GFP (Bax); pYES2–SMS1, pGilda-Bax and p425GAL1ATG–GFP (SMS1α/Bax); p425GAL1–SMS1 β-GFP, pGilda-Bax and pYES2 (SMS1β/Bax); and p425GAL1–SMS1β–GFP, pYES2–SMS1 and pGilda-Bax (SMS1α/SMS1β/Bax). Yeast cells were grown to saturation in YNB glucose containing media, serially diluted and 10 μl of each dilution were spotted onto selective nutrient agar plates containing either glucose (GLU) or galactose (GAL) to induce *BAX*, *SMS1α* or *SMS1β–GFP* expression. Photographs of the resultant plates are shown after 3–4 days of incubation at 30 °C. (B) Levels of SMS1β–GFP protein in different transformants was determined in glucose (GLU) or galactose (GAL) grown cells by Western blot analysis using GFP specific antibody. SMS1β–GFP was detected only in galactose grown cells harboring p425GAL1–SMS1β–GFP. Arrow depicts the location of the SMS1β–GFP protein.

was expressed as GFP fusion. Since no antibodies are available for SMS1, this allowed us to follow the expression of *SMS1β* using GFP antibodies. In addition, the *SMS1β–GFP* fusion is expressed under the control of the *GAL1* promoter since it allows us to induce its expression by the addition of galactose. Using a GFP specific antibody, we detected a 53 kDa band corresponding to the appropriate size for the SMS1β–GFP protein only in galactose grown cells containing the plasmid encoding the *SMS1β–GFP* gene fusion (Fig. 6B). This suggests that the inability of *SMS1β* to affect function of SMS1α is not due to its inability to be expressed in yeast. Although the GFP tag may interfere with SMS1β function, numerous studies have shown that such a tag does not commonly interfere with protein function (Li et al., 2002; Somerville et al., 2003).

4. Conclusions

The *SMS1* gene was found to consist of 16 different exons that are alternatively spliced to yield 4 different transcripts and 3 different proteins. Given that SMS is an important enzyme in sphingolipid metabolism which plays a central role in regulating the levels of the pro-apoptotic ceramide and the mitogenic DAG (Hannun and Luberto, 2004), the complexity of the *SMS1* gene suggest that a number of different processes are involved in regulating the expression of this gene and protein.

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GTP-bound G_α subunits. There appears to be distinct but overlapping specificity of the different RGSs for the different G_α-proteins [5,7,8]. In addition, most of the evidence suggests that the R4 RGSs cannot act as a GAP for $G_{\alpha s}$ subunits. Nevertheless, a number of these RGSs can block signalling from $G_{\alpha s}$ coupled GPCRs by acting as an effector antagonist and effectively blocking $G_{\alpha s}$ mediated activation of effectors such as adenylyl cyclase [9]. Individual R4 RGSs also show distinct receptor specific preferences in their ability to inhibit signalling from different GPCRs [10]. This specificity appears to be determined by the ability of the different R4 RGSs to either bind to specific subsets of GPCRs or to bind to specific GPCR binding adapter proteins [11]. In spite of the absence of definite recognizable motifs, the N-terminal non-RGS portions of these proteins have been implicated in mediating their receptor specificity and their ability to function as effector antagonists [11, 12]. Thus although R4 RGSs are small proteins, their ability to interact with a number of other proteins in addition to their interaction with G_α subunits is critical for their function as modulators of GPCR signalling [13].

Multiple protein-protein interactions are a common theme for most signal transduction cascades including GPCRs [14]. In effect, GPCRs and their associated proteins are now known to assemble into large protein complexes [15, 16]. In addition to interacting with a wide range of different proteins, GPCRs also form homo- and hetero-oligomers [17]. Although RGSs are members of the GPCR containing complexes, the entire repertoire of proteins that are capable of interacting with the R4 RGSs is not known [11, 13].

We have previously generated specific RGS5 antiserum and we have shown that this protein migrates as 23 kDa protein that is abundantly expressed in cardiac and skeletal muscle [18]. Here we continue our analysis of the RGS5 protein and we report a new binding partner for RGS5. In effect, we demonstrate that RGS5 can be detected as a dimer by western blot analysis in both yeast and cultured mammalian HEK293A cells that overexpress RGS5. Using a series of other experimental approaches including the expression and analysis of GFP tagged RGS5 in yeast as well as two hybrid assays, we provide evidence that RGS5 does indeed interact with itself. We also demonstrate that formation of the RGS5 dimer may have a regulatory role since we observe that the dimer is longer lived than the monomer. Thus our results suggest that the dimerization of R4 RGSs, like RGS5, may provide an additional layer of complexity involved in regulating GPCR signalling complexes.

Materials and Methods

Plasmids

To make the RGS5-GFP fusion, RGS5 was amplified using the following forward 5'-ACA CTA GTC AAA CAA TGT GCA AAG GAC TTG CAG CTT TG-3' and reverse 5'-AGA AGC TTG CTT GAT TAA CTC CTG ATA AAA TCT-3' oligos with p423GAL1 RGS5 as a template [19]. The PCR product was subcloned as a *Spe* I-*Hind* III fragment into p426GAL1-GFP [20] to generate plasmid p426GAL1-RGS5-GFP encoding an in-frame RGS5-GFP protein fusion. The plasmids encoding the different GPCR C-tails fused to GFP were constructed by first amplifying the receptor C-tails using reverse transcribed RNA that was obtained from mouse tissues (brain, testes or heart) and the following oligos: SST_5: forward: 5'-AAC TAG TGG CAA ACA ATG TCT GAC AAC TTC CGC CAG AGC-3', reverse: 5'-AGA AGC TTG CAG CTT GCT GGT CTG CAT AAG-3'; β_2AR: forward: 5'-CTA GTC AAA CAA TGA GTC CAG ATT TCA GGA TTGC C-3', reverse: 5'-AAG CTT GCA GTG GCA GGT CAT TTG TAC TAC A-3'; CB_1: forward: 5'-ACT GTC AAC AAT GAA GGA CCT GAG ACA TGC TTTC CGC-3', reverse: 5'-AAG CTT GCA GAG CCT CGG CAG ACG TGT CTG T-3'; and LPA_4: forward: 5'-ACT AGT CAA ACA ATG TAC TTC ACT CTT GAA TCC TTT CAG-3', reverse: 5'-AAG CTT GGA AGG TGG ATT CCA GCA TTA A-3'. The amplified products were subcloned into p426GAL1-GFP as described above for RGS5. The yeast expression plasmid p425GAL-RGS5 and the mammalian expression plasmid for RGS5 were previously described [18, 19]. p426GAL1-ATG-GFP expressing GFP alone under the control of the galactose inducible *GAL1* promoter was previously described [20].

Yeast cells

The *Saccharomyces cerevisiae* strain BY4741-6055 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sst2Δ::G418^R*) was used for these studies (Research Genetics). Yeast cells were routinely grown on synthetic minimal media consisting of Yeast Nitrogen Base (YNB) containing 2% glucose supplemented with the appropriate amino acids and bases [21]. Plasmids were introduced into yeast using lithium chloride and the resultant transformants were selected and maintained by the omission of the appropriate supplements from the growth media (leucine for p425GAL1 and pACT2, uracil for p426GAL1, and tryptophan for pLexA-dir vectors). Glucose was replaced with 2% galactose and 2% raffinose to induce *GAL1* dependent expression. The semi-quantitative halo assay was used to assess the ability of RGS5-GFP to function as an RGS essentially as previously described [19, 22]. Sterile filter disks containing 1000 and 3000 pmoles of α-factor, were placed onto the top of a lawn of yeast cells and the plates were subsequently incubated at 30°C for 3 to 4 days.

Mammalian cultured cells

HEK 293A cells were grown in DMEM media containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (pen-strep). The cells were transfected with pcDNA alone or with pcDNA plasmid expressing RGS5 using lipofectamine (Invitrogen).

Protein extraction and western blot analysis

Soluble protein was extracted from yeast by directly heating the NaOH treated cells to 95°C in SDS-PAGE loading buffer as previously described [20]. Soluble protein was extracted from transiently transfected HEK293A cells using ice cold lysis buffer [23]. Equal amounts of soluble protein were separated by SDS-PAGE, transferred to nitrocellulose membrane and challenged with the previously described RGS5 anti-serum [18] or the commercially available GFP or LexA anti-sera (Santa Cruz Technology). HRP-conjugated secondary anti-serum were used and signals were subsequently detected with ECL plus (AmershamBioscience) followed by exposure to X-ray film (Kodak X-Omat). The relative expression levels were determined using Multi-Analyst software (BioRad). Unless otherwise indicated, western blots were repeated at least three times with similar results.

Two hybrid analysis

Plasmid pLexA-dir was used as the bait vector and plasmid pACT2 was used as the prey vector [24]. Human RGS5 was prepared for cloning into the prey vector by PCR amplification using the respective forward 5'- GCT TAC CCA TAC GAT GTT CCA GAT TAC GCT ATG TGC AAA GGA CTT GCA GCT-3 and reverse 5'-ACT TGC GGG GTT TTC AGT ATC TAC GAT TCT TAC TAC TTG ATT AAC TCC TGA TA-3' oligonucleotides. The N-terminal region of RGS5 was prepared for cloning into the bait vector by PCR amplification using the following respective forward 5'-GTT GGG GTT ATT CGC AAC GGC GAC TGG CTG ATG TGC AAA GGA CTT GCA GCT -3' and reverse 5'-TCA TAA GAA ATT CGC CCG GAA TAG CTG GCA GGG CCT CGT CCA GCG AGG T-3' oligonucleotides. The C-terminal RGS box regions of RGS5 was prepared for cloning into the bait vector by PCR amplification using the following respective forward 5'-GTT GGG GTT ATT CGC AAC GGC GAC TGG CTG CAG TGG CGT GAT TCC CTG GAC-3' and reverse 5'-TCA TAA GAA ATT CGC CCG GAA TTA GCT GGT TAC TAC TTG ATT AAC TCC TGA TA-3' oligonucleotides. The PCR products were subcloned by recombination in yeast using *Eco*RI digested two hybrid vectors [25]. Different combinations of the plasmids were transformed into the yeast strain DSY-1 (*MATa his3Δ200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)$_4$-HIS3 URA3::(lexAop)8-lacZ GAL4*). Freshly saturated cultures of the transformed were serially diluted and spotted onto YNB glucose agar plates with and without histidine [26].

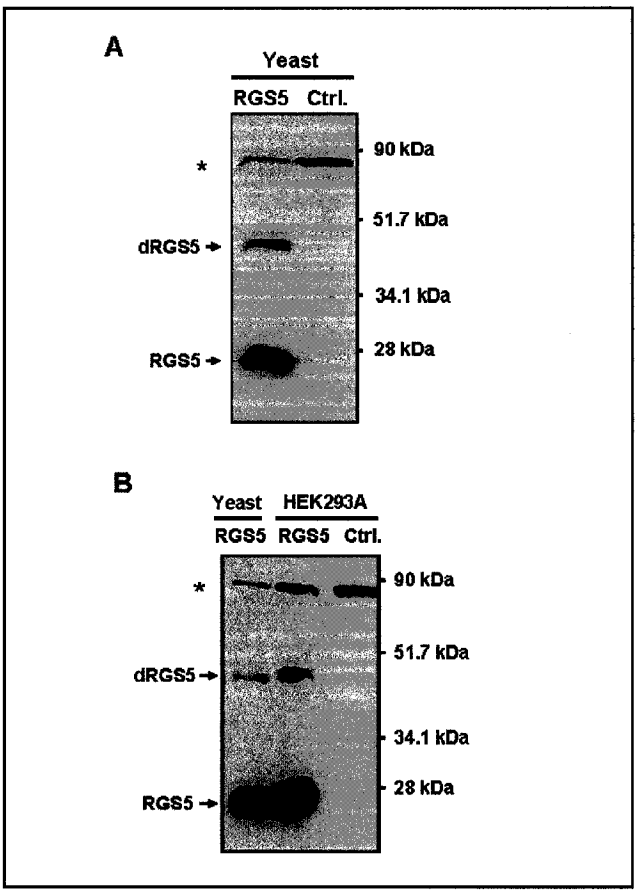


Fig. 1. Detection of a RGS5 dimer in cells overexpressing RGS5. (A) Western blot analysis of protein extracts prepared from control (Ctrl.) yeast cells or from yeast cells expressing RGS5 (RGS5). (B) Western blot analysis of protein extracts from HEK 293A cells. Cells were transfected with either the mammalian expression plasmid pcDNA (Ctrl.) or a pcDNA expressing a hRGS5 cDNA (RGS5). As a control, 0.5 μg of protein extract from yeast cells expressing hRGS5 was included on the gel (left lane). In both A and B, the extracts were challenged with RGS5 anti-serum. In addition the location of bands corresponding to the sizes of RGS5 and putative RGS5 dimers (dRGS5) are indicated. A non-specific band of approximately 87 kDa is apparent in the extracts prepared from both mammalian and yeast control cells and is indicated by an "*". Similar results were obtained in four different experiments.

Results

Identification of an RGS5 dimer

Human RGS5 is a 181 residue protein that belongs to the R4 RGS subfamily. It has a calculated molecular weight (MW) of 20.9 kDa and we have previously demonstrated that it migrates as a single band of 23 kDa when detected by western blot analysis [18]. The same 23 kDa band was detected in yeast and HEK293A cells overexpressing human RGS5 and in tissues endogenously expressing RGS5. Here we demonstrate that in addition to recognizing the 23 kDa RGS5 protein, our affinity purified RGS5 anti-serum also recognizes proteins with apparent MWs of 87 and 42 kDa in extracts prepared from yeast cells expressing RGS5 (Fig. 1A). The 87 kDa band was also detected in extracts prepared from control yeast cells suggesting that it represents a non-specific

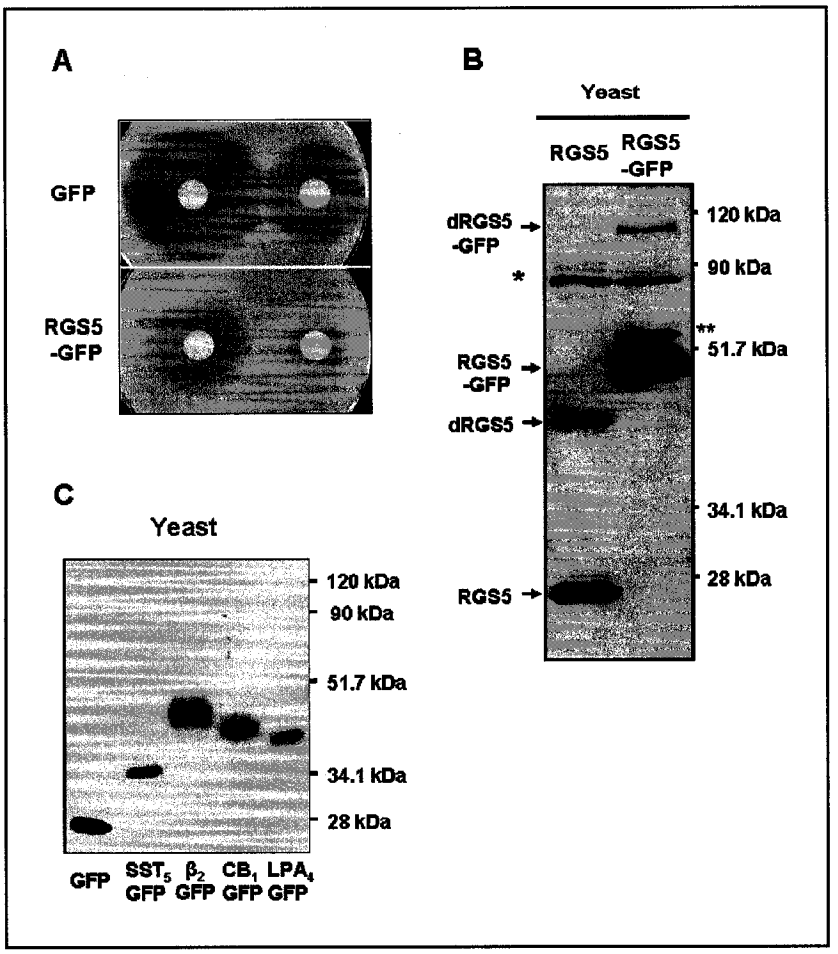


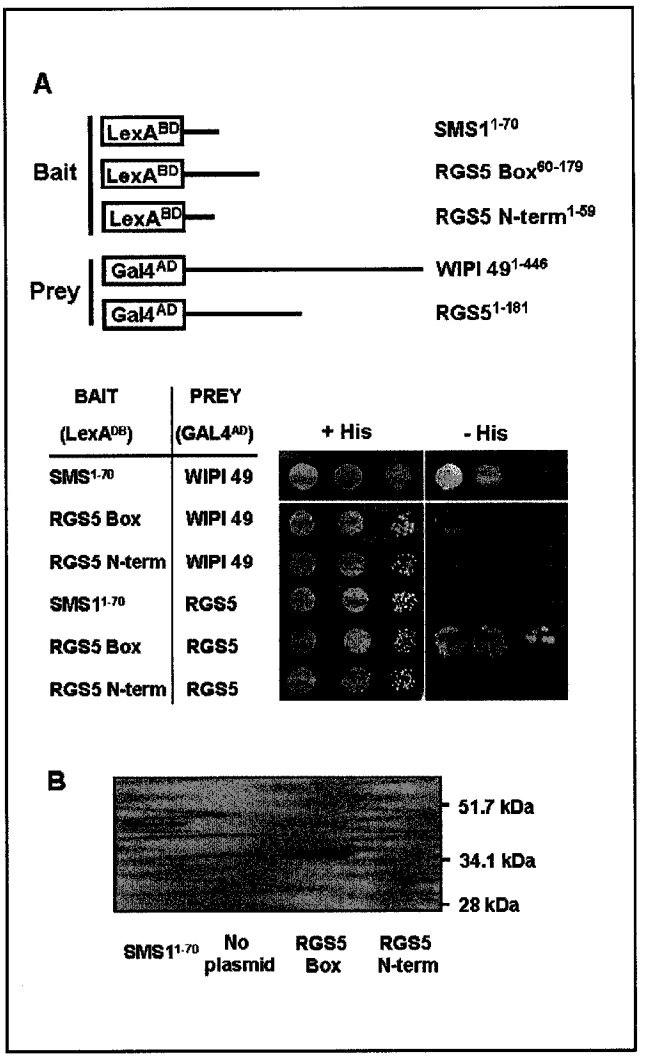
Fig. 2. Analysis of a GFP tagged RGS5 gene in yeast. (A) Halo assays were performed on yeast transformants harbouring plasmid p426GAL1-ATG-GFP (GFP) or p426GAL1-RGS5-GFP (RGS5-GFP). Sensitivity to the growth inhibitory effects of the yeast GPCR agonist is proportional to the zone of no growth surrounding the α-factor containing filters. (B) Western blot analysis using RGS5 anti-serum with extracts prepared from yeast cells expressing RGS5 or RGS5-GFP. The locations of the RGS5 and RGS5-GFP monomers and the putative dimers (dRGS5 and dRGS5-GFP) are shown. An "*" denotes the non-specific 87 kDa band while a double asterisks (**) denotes a band that is reproducibly observed but whose origin remains unknown. Molecular weight markers in kDa are shown on the right of the blot. (C) Western blot analysis, using GFP anti-sera, of extracts prepared from yeast cells expressing GFP alone and GFP fused to the C-tails of different GPCRs (SST_5, somatostatin receptor 5, 56 residues, GenBank accession # NM_001053; β_2AR, β_2-adrenergic receptor 2, 90 residues, accession # BC032883; CB_1, cannabinoid receptor1, 70 residues, accession # NM_007726; and LPA_4, lysophosphatidic acid receptor 4, 57 residues, accession # NM_020028). All western blots were performed at least three times with similar results.

cross-reactive protein. In contrast, the 42kDa protein appears to be a modified form of RGS5, since it is not seen in control yeast cells that do not overexpress RGS5 (Fig. 1A). Because the 42 kDa protein corresponds to the approximate MW of two RGS5 proteins, we hypothesized that it may represent a RGS5 dimer. The putative RGS5 dimer is also detected in extracts prepared from HEK293A cells that are overexpressing RGS5 (Fig. 1B). This later result suggests that the putative dimer is not an artifact of overexpressing RGS5 in the heterologous yeast cell. Using the same affinity purified anti-sera, we previously reported that RGS5 protein could only be detected in skeletal and cardiac muscle tissue and cell lines [18]. We were unable to detect the putative RGS5 dimer in extracts prepared from these tissues or cells because of the large number of cross-reactive proteins that were recognized by the anti-serum (not shown).

Analysis of an RGS5-GFP fusion in yeast

R4 RGSs are known to interact with other proteins [4, 13]. Although the interaction of RGS5 with G_α proteins is likely to be transitory, the strength of its interaction with other proteins such as the chaperone 14-3-3 is not well known [13]. Since many yeast and human proteins share similar sizes and function, it remains possible that the putative RGS5 dimer that we observe actually represents a dimer that forms between RGS5 and another protein. To further investigate the possibility that we observe an RGS5 dimer, we constructed an RGS5-GFP fusion gene in order to express a GFP tagged RGS5 protein in yeast. Given that the RGS5-GFP protein has a calculated MW of 47.7 kDa, we should be able to detect a 95 kDa protein if RGS5-GFP does indeed form a dimer with itself. As a first step towards characterizing the RGS5-GFP fusion protein, we used a yeast mutant lacking

Fig. 3. Analysis of RGS5-RGS5 interaction using the yeast two hybrid system. (A) A schematic diagram depicting the fusion proteins used in this study is shown at the top. Yeast cell were transformed with different combinations of bait (BAIT) and prey (PREY) expressing plasmids as indicated. Cultures of the different transformants were serially diluted and aliquots were spotted onto nutrient agar with (+His) and without (-His) histidine. $SMS1^{1-70}$ represents the N-terminal 70 residues of sphingomyelin synthase (GenBank accession no. AY509044) and WIPI49 is a two hybrid clone that was found to interact with $SMS1^{1-70}$ in a two hybrid screen of a mouse cardiac cDNA library (Dualsystems Biotech AG). Growth in the absence of histidine (-His) indicates that an interaction is occurring between the bait and the prey tested. Similar results were obtained in 3 different experiments using two different groups of yeast transformants. (B) Extracts prepared from yeast cells expressing the different LexA-bait protein fusions, described in A, were analyzed by western blot using a specific LexA anti-serum.



its RGS-encoding *SST2* gene to determine if RGS5 retained RGS function when fused to GFP. *Sst2* yeast cells are hypersensitive to GPCR stimulation and are consequently hyper-responsive to the growth inhibitory effects of the GPCR agonist á-factor. We and others have shown that a number of different mammalian RGSs, including RGS5, can inhibit endogenous GPCR signalling and confer an increased resistance to α-factor when expressed in yeast [1, 4, 19, 27]. Using the halo assay, we show that cells transformed with the control plasmid p26GAL1-ATG-GFP (which expresses GFP alone) show a significant zone of no growth around the filter discs containing α-factor indicating a strong GPCR mediated response (Fig. 2A). In contrast, expression of RGS5-GFP attenuated the growth inhibitory effects of α-factor (Fig. 2A, bottom plate). These results indicate that RGS5, like a number of other RGSs, can be fused to GFP and retain its ability to function as an RGS [20, 28, 29].

Western blot analysis was then carried out using extracts prepared from yeast cells expressing RGS5 or RGS5-GFP. As seen in Figs. 1A and 2B, we observe 2 specific bands in cells expressing RGS5 using the RGS5 anti-serum. The 23 kDa band represents the RGS5 monomer while we have named the 42 kDa band dRGS5 to reflect the fact that it may be a RGS5 dimer. Both of these bands are absent in cells expressing RGS5-GFP. Instead, we observe a prominent band having a calculated MW of 50 kDa, which corresponds closely to the calculated MW of 47.7 kDa for RGS5-GFP (Fig. 2B). In addition, a band having an estimated MW of 100 kDa is also present. The size of the band, which we have named dRGS5-GFP, has the appropriate MW to represent a RGS5-GFP dimer. These results strongly suggest that the observed 42 kDa that cross-reacts with the RGS5 anti-sera is indeed an RGS5 dimer.

GFP has been reported to be capable of self-dimerizing under some conditions [30, 31]. In order to rule out the possibility that the observed RGS5-GFP doublet is due to GFP itself, we performed a western blot analysis using protein extracts prepared from yeast transformants expressing GFP alone or GFP fused to the C-terminal regions of a number of different GPCRs (Fig. 2C). Using specific GFP anti-serum, a single band corresponding to the appropriate MWs of GFP or the GFP fusions was detected (Fig. 2C). Thus GFP does not form a dimer that is detectable by western blot analysis when expressed in yeast.

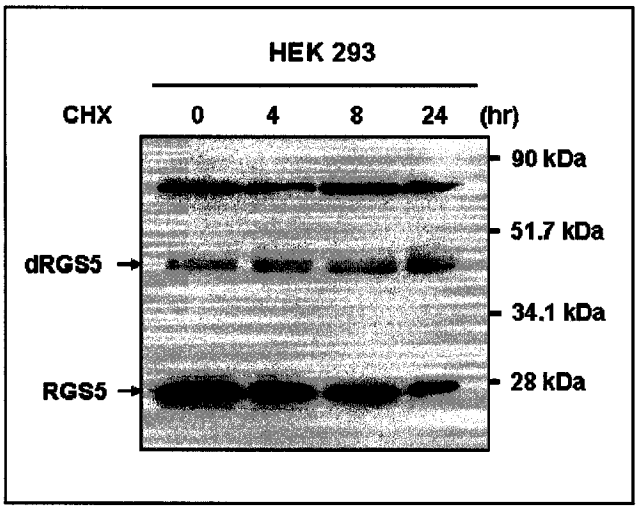


Fig. 4. Analysis of the stability of the RGS5 monomer and dimer. Identical cultures of HEK293A cells expressing hRGS5 were treated with cycloheximide (CHX, 50 μg/ml). Samples were harvested prior to the addition of CHX (0) and after 4, 8, and 24 hours (hr). Extracts were prepared and analyzed for RGS5 expression by western blot. Similar results were obtained in two independent experiments.

RGS5-RGS5 interaction by two-hybrid analysis

Although the previous results clearly indicate that RGS5 can form a dimer, it remains that this dimer may be a methodological artifact. We therefore made use of the yeast two-hybrid system as a different approach to determine if we could detect RGS5-RGS5 interaction [24]. Since the yeast strain used has a LexA-*HIS3* fusion reporter gene, histidine prototrophy indicates that a given bait and prey are physically interacting. The spot assay was therefore used to assess growth in the absence of histidine of yeast cells transformed with different combinations of bait and prey vectors that express the different protein fusions shown in Fig. 3A. As a control, we show that all yeast transformants are capable of growth when the growth media is supplemented with histidine (Fig. 3A). As a positive control, we made use of the fact that WIPI49 has been previously shown to interact with the N-terminus of sphingomyelin synthase (unpublished observation). Yeast cells harbouring the bait plasmid expressing $SMS1^{1-70}$ [26] and the WIPI49 expressing prey vector are positive for growth in the absence of histidine (Fig. 3A). In addition to these plasmids, we transformed yeast with different bait plasmids expressing the N-terminus ($RGS5^{1-59}$) or the C-terminal RGS box ($RGS5^{60-181}$) of RGS5 and prey vector expressing the entire RGS5 coding sequence. Of the different combinations tested, only the RGS5 box and RGS5 were found to interact (Fig. 3A). Given that RGS5 did not interact with $SMS1^{1-70}$ or that the RGS5 box did not interact with WIPI49, the observed RGS5 Box interaction with RGS5 appears to be specific (Fig. 3A). It nevertheless remained possible that the negative results demonstrating that the N-terminus does not interact with RGS5 is due to the $RGS5^{1-59}$ bait protein not accumulating in yeast cells. We therefore used western blot analysis to show that all the bait proteins used in this study, including the RGS5 N-terminal-LexA fusion accumulate in yeast cells (Fig. 3B). Taken together these results provide strong evidence that RGS5 is capable of self-interacting.

RGS5 dimer is more stable than the RGS5 monomer

The levels of many of the R4 RGSs, including RGS5, are regulated in part by post-translational mechanisms [4, 6, 32-34]. We therefore wanted to examine the possibility that the RGS5 dimer may represent a sub-population of RGS5 proteins that are differentially regulated than the monomer. To examine the stability of RGS5, HEK293A transfected with pcDNA-hRGS5 were treated with cycloheximide and samples were harvested at intervals for western blot analysis using RGS5 specific anti-sera. Upon blocking protein synthesis with cycloheximide, the levels of RGS5 monomer were found to decrease with a half life of 20 hours (Fig. 4). In contrast, the RGS5 dimer remained stable with 100% of the protein still present 24 hours after the addition of cycloheximide (Fig. 4). This demonstrates that RGS5 dimer is differentially regulated than the monomer.

Discussion

Members of the R4 RGS sub-family are best known as proteins that can serve as GAPs for subsets of different heterotrimeric Gα proteins [4-8, 35]. Our understanding of their involvement in cellular processes such as their ability to function as effector antagonists as grown alongside our understanding of the diversity of proteins that interact with the R4 RGSs [9,13]. The list has grown to include chaperones, GPCRs, effectors and adapter proteins. Here we add to this list by providing the first report that describes the dimerization of a member of the R4 RGS sub-family. RGS5 dimerization was first detected as a protein having the MW of 2 RGS5 proteins by western blot analysis in extracts prepared from both yeast and HEK 293 cells overexpressing RGS5 (Fig. 1).

Detection of a RGS5-GFP dimer in the extracts prepared from yeast cells expressing a functional RGS5 cDNA tagged with GFP served to confirm that RGS5 is capable of dimerizing (Fig. 2). The ability of the RGS5 and RGS5-GFP dimers to remain intact after SDS-PAGE suggests that the observed RGS5-RGS5 dimer is resistant to detergent and reducing agents that are used to prepare the samples. Although this may reflect covalent attachment, non-covalent interaction may also lead to such strongly attached dimers. The situation is reminiscent of other non-covalently attached dimers, such as GPCRs, that have been shown to remain attached under such stringent conditions [36]. The nature of the RGS5-RGS5 dimer remains at present unknown.

Yeast two hybrid analysis was used to confirm that that RGS5 was capable of self-interacting (Fig. 3). Our results suggest that the RGS5 interacting region is present within the RGS box. These results are somewhat surprising since the RGS box is thought to be the site of interaction with G_{α}-proteins while the N-terminal region has been shown more versatility since it can interact with a number of different proteins including GPCRs, effectors and some adapter proteins [11, 13]. The involvement of the RGS box suggests that RGS5-RGS5 interaction may have a regulatory role. For example a number of R4 RGSs interact with 14-3-3 and this interaction may serve to inhibit RGS GAP activity [13]. It would be interesting to determine if RGS5 dimerization serves a role in modulating the GAP activity of RGS5. We previously demonstrated that RGS5 protein is abundantly expressed in cardiac and skeletal muscle [18]. Basal levels of RGSs are known to limit GPCR responsiveness while decreases in the basal levels of RGSs increase GPCR responsiveness [4, 6]. Alterations in the activity of RGS5 by dimerization may therefore serve to regulate a subset of the large number of GPCRs that are known to be expressed in cardiac and skeletal muscle [37, 38].

Analysis of the stability of RGS5 ectopically expressed in HEK293 cells suggested that the RGS5 monomer has a shorter half-life than the dimer (Fig. 4). These results are consistent with previously published reports showing that RGS5 has a relatively short half-life [32-34]. The N-end rule ubiquitin dependant pathway has been shown to be involved in degrading RGS5 [33, 34]. Thus dimerization of RGS5 appears to prevent the rapid degradation of RGS5 by as of yet unknown process that may simply involve the masking of the N-terminal region of RGS5. RGS5 levels, as observed for a number of other RGSs, are known to be increased in response to GPCR stimulation [4, 8, 18]. We nevertheless find no difference in the levels of the RGS5 monomer in HEK cells overexpressing RGS5 that are stimulated with GPCR agonists (not shown). Although the differential stability of the monomer versus the dimer is consistent with our results, it nevertheless remains possible that the RGS5 monomer is not lost but instead it could be converted to the dimeric form. Experiments using proteosome inhibitors are underway in order to further examine these possibilities.

Although the possible significance of RGS dimerization is not known, it is worthwhile mentioning that the significance of GPCR dimerization was not well understood when it was first reported [36]. Nevertheless, today it is quite clear that GPCR-GPCR interaction is a widespread phenomenon that plays critical roles in multiple aspects of the biology of these receptors [17]. The implications for RGS5 function in regulating GPCR signalling as well as the possible dimerization of other R4 RGSs will be questions of great interest. In this context, it is interesting to note that purified RGS4 appears to form a dimer in vitro [33]. Although RGS4 and RGS5 along with RGS8 and RGS16 may be a subfamily R4 RGSs [19], these results suggest that RGS dimerization may be a common phenomenon.

Abbreviations

GFP (Green fluorescent protein); GPCR (G-protein Coupled Receptor); RGS (Regulator of G-protein Signalling); SMS (Sphingomyelin synthase).

Acknowledgements

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Appendix III. Certificates