UP REGULATION OF CAVEOLIN-1 DURING H_2O_2 INDUCED OXIDATIVE STRESS

by

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ABSTRACT

Caveolae ("little caves") are 50 -100 nm invaginations seen on the plasma membrane of most cells. Caveolin-1, the marker protein of caveolae in most tissues, is the structural and functional unit of caveolae.

Previous studies have demonstrated the tumor suppressor capability of caveolin-1. Our lab has already shown that expression of caveolin-1 induces premature senescence in cells, possibly including those with malignant potential. We proposed that this might explain the tumor suppressor function of caveolin-1. We have further shown that oxidative stress induces premature senescence through up regulation of caveolin-1. To further elucidate the molecular mechanisms underlying this process, we used H_2O_2 as a model to generate stress induced premature senescence (SIPS) and examined the response of caveolin-1 promoter under these conditions.

Constructs with serially truncated segments of the mouse caveolin-1 gene promoter linked to a luciferase reporter gene were made and luciferase assays were carried out. These experiments demonstrated that the critical regions lay in two segments: -222/-372 and -91/-150 of the promoter region. A consensus Sp1 binding sequence was identified within each deleted segment. Gel shift analysis of protein binding from nuclear extracts to these caveolin promoter DNA sequences confirmed that transcription factors were binding to the Sp1 consensus elements as

part of the transcriptional response to H_2O_2 induced senescence. Further deletion mutagenesis of the individual Sp1 consensus sites confirmed the identity of the transcription factor to be Sp1. These findings suggest that Sp1 mediates oxidant induced up regulation of caveolin-1 expression. In subsequent experiments, we examined the effect of inhibitors of p38 Mitogen activated protein kinase pathway on the levels of caveolin-1 expression during SIPS by western blot and luciferase assay. We found that this pathway plays a direct role in the up regulation of caveolin-1 during SIPS, possibly through modification of Sp1 to increase its activity.

Public Health Importance: Cancer is a leading public health concern. Cav-1 has been shown to be a tumor suppressor gene involved in a large number of human tumors. Induction of premature senescence in cells with malignant potential is thought to be a vital tumor suppressor function. Our study aims to define the tumor suppressor capacity of Cav-1 by elucidating the pathway by which it induces premature senescence.

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LIST OF ABBREVIATIONS

β- gal: beta galactosidase

Ab: antibody

ATP: adenosine triphosphate

Cav-1: caveolin-1

Cav-2: caveolin-2

Cav-3: caveolin-3

CBD: caveolin binding domain

ChIP: Chromatin immunoprecipitation

CMAD: carboxy terminal membrane anchoring domain

CPRG: chlorophenol red-β-galactopyranoside solution

CSD: caveolin scaffolding domain

Ctl: control

DMEM: Dulbecco's modified eagle medium

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DTT: dithiothreitol

EDTA: ethylenediaminetetraacetic acid

EMSA: electrophoretic mobility shift assay

eNOS: endothelial nitric oxide sythase

ERK: extra cellular signal regulated kinases

GTP: guanosine triphosphate

GTPases: guanine triphosphatases

HDL: high density lipoproteins

HPV: human papilloma virus

MAPK: mitogen activated protein kinase

MEK: mitogen activated protein kinase kinase

mRNA: messenger ribonucleic acid

p38 MAPK: p38 mitogen activated protein kinase

PCR: polymerase chain reaction

PIP1: phospho inositol monophosphate

PIP2: phosphor inositol diphosphate

RNA: ribonucleic acid

SDS-PAGE: sodium dodecyl sulphate- polyacrylamide gel electrophoresis

SIPS: stress induced premature senescence

Sp1: specificity protein 1

SR-B1: sterol receptor- B1

TAE: tris, acetic acid, EDTA

TBE: tris, boric acid, EDTA

TBS: tris buffered saline

TBS-T: tris buffered saline-tween

1. INTRODUCTION

Caveolae are flask shaped invaginations seen on the surface of most cells. (8, 9) They have a distinct group of marker proteins called caveolins. (8) Hetero oligomers of caveolin-1 (Cav-1) and caveolin-2 (Cav-2) are found in the caveolae of most cells. (16) Caveolae have been shown to have a number of functions including acting as docking sites for numerous cell signaling molecules. (51 – 53) Cav-1 has been shown to negatively modulate these molecules and thus have a general inhibitory effect on their downstream cell signaling pathways. (54 – 56) These pathways include those involved in cell growth and proliferation. Furthermore, a number of studies, both *in vitro* and *in vivo* show that Cav-1 could be a tumor suppressor gene. (103 – 124) Our lab has previously shown that this tumor suppressor function of Cav-1 could be explained by its ability to induce premature senescence under conditions of stress - stress induced premature senescence. It is well known that when cells are subjected to external stress (UV radiation, etc.) or internal stress (free radicals etc), a minor fraction turn malignant. We proposed that Cav-1 induces SIPS in these cells with malignant potential and that the cells that escape this fate continue proliferation, causing tumors. We had showed that Cav-1 is up regulated when NIH 3T3 cells are subjected to oxidative stress and induces premature senescent phenotype. However, the exact molecular pathways leading to the up regulation of Cav-1 during SIPS remain unknown. (138) The present study aims to elucidate the pathways leading to up regulation of Cav-1 during oxidative stress induced premature senescence.

2. SPECIFIC AIMS

1) To delineate the cis-regulatory elements of Cav-1 gene involved in SIPS
2) To identify the transcription factor(s) binding to the regulatory elements of Cav-1 gene in oxidative stress
3) To determine the response of Cav-1 promoter region to antioxidants during oxidant induced SIPS
4) To identify the pathways involved in up regulation of Cav-1 during oxidative stress.

3. LITERATURE REVIEW

Traditionally, the plasma membrane was thought to be a "fluid mosaic" as proposed by Singer and Nicholson in 1972. (1) It was thought that the plasma membrane was made of homogenously distributed lipids in which proteins were more or less uniformly distributed. These proteins were thought to be free to move about laterally in the membrane. (2,3) However, the present consensus is that the plasma membrane is not uniform but has heterogeneous regions – i) the "liquid disordered state" forming the bulk of the membrane existing in a relatively fluid and less rigid state and ii) specialized domains existing in a more rigid "liquid ordered state." These specialized domains are called lipid rafts. (4, 5) Lipid rafts, when compared to the rest of the plasma membrane are rich in i) glycosphingolipids; ii) sphingomyelin; iii) cholesterol and iv) several proteins. (6, 7) Caveolae are a distinct subset of flask shaped lipid rafts. (8) Although caveolae and lipid rafts have a similar biochemical composition, they differ in the proteins that reside in them. In particular, caveolae have the coat protein called caveolin which has been shown to be essential for the formation of caveolae. (8)

3.1. CAVEOLAE

The word caveolae literally means "little caves." Caveolae are 50-100 nm invaginations seen on the plasma membrane on most cell types. Caveolae are almost ubiquitous in distribution and are found on the surface of most differentiated cell types. (9) Endothelial cells, type 1 pneumocytes and muscle cells are particularly abundant in caveolae. (9) As already discussed, caveolae have a distinct group of proteins called caveolins unique to them as shown in figure 1. This figure also shows that caveolae are rich in the lipids that are found other kinds of lipid rafts. Caveolin proteins are the structural and functional units of caveolae. (10)

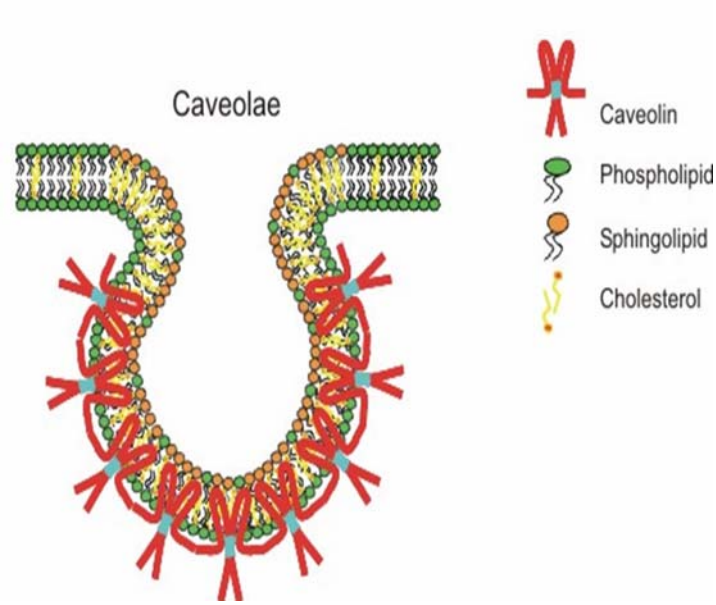

Figure 1
Structure of caveolae

Caveolae are flask shaped invaginations of the plasma membrane. They are rich in phospholipids, sphingolipids, and cholesterol. Caveolin-1 is found exclusively in caveolae and acts as a scaffolding protein (Modified from Razani et. al, 2002, Ref.163)

3.2. CAVEOLIN PROTEINS

To date, three members of the caveolin family have been identified. Caveolin-1 (Cav-1) was the first member to be identified. (11) Later, caveolin-2 (Cav-2) and caveolin-3 (Cav-3) were also identified. (11, 12) Caveolin-1 and 2 have isoforms. (α and β for cav-1 and ά, β and γ for cav-2). However, the functional differences between the various forms are not yet known. (13, 14, 15) All the three genes of this family are very highly conserved through lower animals and have significant homology. (16)

Cav-1 and Cav-2 are co expressed in most tissues having caveolae except striated muscle and cardiac muscle. (16) In these tissues (skeletal and heart muscle), Cav-3 is expressed and takes over the functions of Cav-1 and Cav-2. (17, 18) However, recent studies have shown that the

levels and type of caveolin proteins expressed varies with degree of cell differentiation, especially in muscle cells. (17, 18) Cav-1 is sufficient and necessary for the formation of caveolae. (14, 19) Cav-2 is insufficient for the formation of caveolae. In fact, Cav-2 is incapable of independent existence without Cav-1 and is always co-expressed with Cav-1 and found along with Cav-1 in tissues expressing these genes. (20) In tissues lacking Cav-1 but having Cav-3, Cav-3 largely plays the role of Cav-1 and is sufficient to drive the formation of caveolae. (21)

3.2.1. CAVEOLIN -1

3.2.1.1. STRUCTURE

Cav-1 is made of 178 amino acids and has a molecular weight of 22 kDa. (11) During post translational modifications, it is palmitoylated on three cysteine residues (at positions 133, 143, and 156). (22) As already discussed, caveolin-1 is localized to the plasma membrane in the cell. Both the NH_2 terminal and the COOH terminal of this protein face the cytoplasm. The intervening hydrophobic (i.e. lipid soluble) trans-membrane domain between these two termini forms a hairpin loop in the plasma membrane. This hairpin loop however, does not span the plasma membrane completely giving cav-1 has a unique structure. (23, 24)

Cav-1 has five distinct domains (Figure 2) which have distinct functions:

i) Trans-membrane domain: (between amino acids 102 and 134). This hydrophobic domain forms the hairpin loop into the plasma membrane as discussed above. Surprisingly, even though this domain traverses the plasma membrane, it has been shown that it is not essential for attachment of caveolin protein to the membrane. This function is carried out by :

ii) NH_2 terminal membrane anchoring domain (NMAD) (between amino acids 82 and 101) and

iii) COOH terminal membrane anchoring domain (CMAD) (between amino acids 135 and 150). (25 - 28) The other domains are :

iv) Oligomerization domain (between amino acids 61 and 101). This domain helps in formation of hetero (Cav-1 and Cav-2) and homo oligomers.(29)

v) Scaffolding domain (CSD, between amino acids 82 and 101). This domain holds and interacts with various proteins, including cell signaling molecules present in the caveolae. (30 - 32)

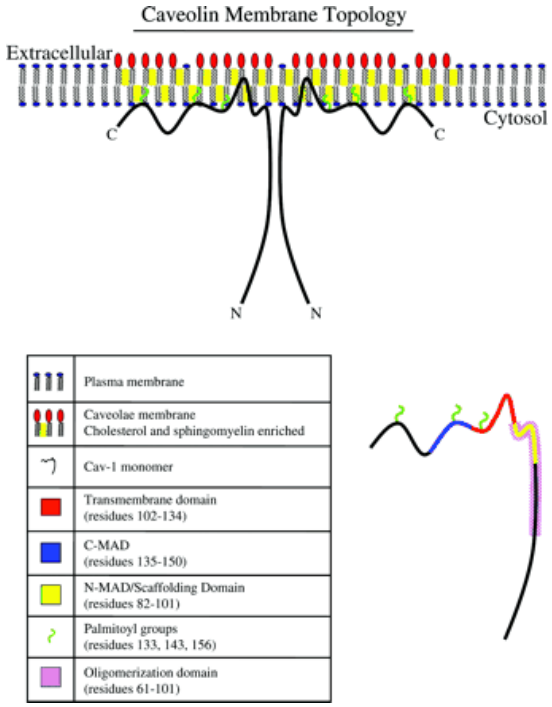

Figure 2

Structure of caveolin-1

Caveolin-1 has the following domains: C-MAD (blue) and N-MAD (yellow) which attach the protein to the membrane, transmembrane domain (red) forming a hair pin loop through a part of the membrane, Oligomerization domain (hashed pink) mediating oligomerization and the scaffolding domain (yellow) through which caveolin-1 interacts with various proteins.. Caveolin-1 is also palmitoylated on three cysteine residues (green; 133, 143, and 156). (Modified from Cohen et. al, 2004, Ref. 164)

3.2.1.2. FUNCTIONS

Caveolin-1 has been shown to have three main functions (Figure 3):

1) Vesicular transport
2) Cholesterol homeostasis
3) Signal transduction.

Each of these functions shall be discussed in detail.

1) Vesicular transport: Since caveolae are cell membrane structures capable of detaching and forming vesicles, one of the earliest functions ascribed to them was transcytosis. Numerous

experiments have shown that caveolae indeed carry out this function in endothelial cells. (33, 34) Studies have also shown that caveolae carry out endocytosis in addition to transcytosis. Numerous molecules have been shown to enter the cell through caveolae including viruses, bacteria, fungi and bacterial toxins. It is thought that upon stimulation, caveolae undergo budding to form vesicles and fuse together. (35 - 37) GTP hydrolysis by dynamin present at the apical regions of caveolae and tyrosine kinase signaling (35 - 39) are thought to be central to this process. Endocytosis via caveolae has been best studied in SV40 virus. It has been shown that the vesicles formed from caveolae form caveosomes that move to the endoplasmic reticulum (40) This pathway bypasses the lysosomes, thus protecting the SV40 virus from the deleterious effects of the lysosomal enzymes. It has been suggested that this pathway could be a prototype for the entry of many other viruses (41). However, other authors have argued that caveolae are relatively stable structures and that endocytosis through caveolae is slow and relatively unimportant (42, 43).

2) Cholesterol homeostasis: Cav-1 and cholesterol have a complex and interdependent relationship. It has been convincingly shown that cholesterol regulates Cav-1 expression. (44, 45) In fact, cholesterol depleting agents cause decreased levels of Cav-1 and flattening of caveolae. (46, 47) Cav-1 in turn, transports cholesterol from the endoplasmic reticulum to the caveolae. Cholesterol thus transported to the plasma membrane i) may diffuse to other parts of the plasma membrane or ii) may be transported on to HDL molecules. This is supported by the fact that SR-B1 receptors (which have been shown to have a role in transfer of cholesterol onto HDL particles) have been identified in caveolae. (48 - 50)

3) Signal Transduction: Initial studies trying to identify proteins other than caveolins that are localized to caveolae showed that a surprisingly large number were signaling molecules. (51, 52)

This list has been growing since. Although it is not known exactly what determines the localization of a particular protein to caveolae, most caveolar proteins have been found to have a common motif called the Caveolar Binding Domain (CBD). (53) The CBD domain is rich in aromatic amino acids and interacts with the CSD of Cav-1. (53) Keeping in view the large number and variety of signaling molecules isolated from caveolae, Lisanti et.al proposed the "Caveolae Signalling Hypothesis." (54, 55) They suggested that localization of components of different pathways resulted in efficient "cross talk" among them. More importantly, they suggested that localization of these molecules to caveolae resulted in better regulation of the downstream pathways. CSD by interacting with the signaling molecules through the CBD has a general inhibitory effect on most pathways. Cav-1 thus inhibits most signaling pathways- these include those involved in cell cycle and cell growth. (54 - 56) The major classes of signaling molecules localized in caveolae are shown in Table 1

Table 1

List of the major groups of signaling molecules localized to caveolae with representative examples.

Class	Examples
G-protein-coupled receptors	β_1, β_2 adrenergic receptors (57, 58)
G-proteins (59)	
Membrane proteins	Insulin receptors (60, 61)
Non- receptor Tyrosine kinases	Src, Fyn (62, 63)
Non-receptor Ser/Thr kinases	MEK, ERK (64)
Cellular proteins/adaptors	PIP, PIP2 (65, 66, 67)
Nuclear proteins	Androgen and Estrogen receptors (68, 69)
Structural proteins	Actin, Annexin II and IV (62, 70 - 72)
Enzymes	eNOS (73-76)
GTPases	H-Ras (77 – 79)

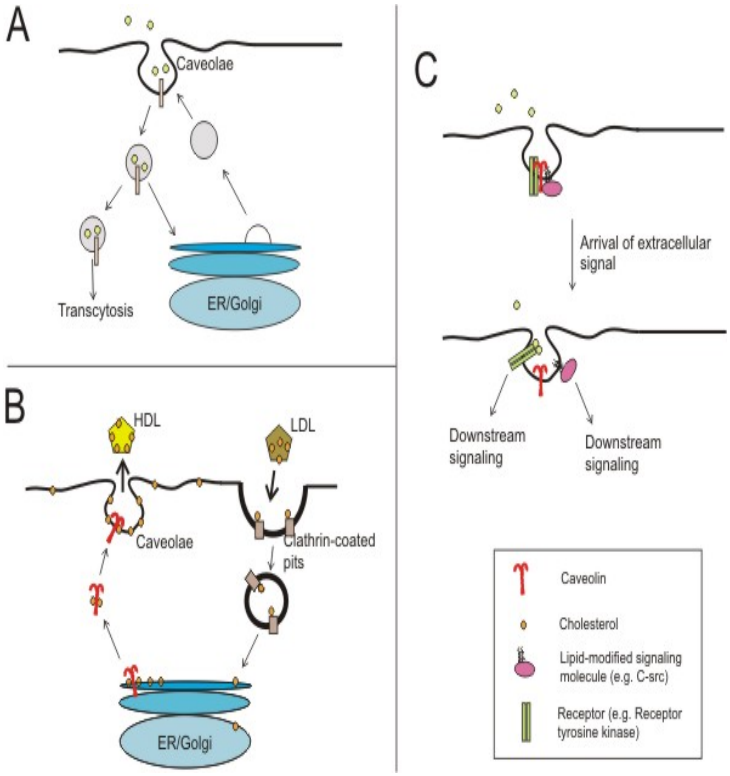

Figure 3

Functions of Caveolin-1

Cav-1 has important roles in vesicular transport (A), cholesterol homeostasis (B) and cell signaling (C) (Modified from Cohen et. al, 2004 Ref. 164)

3.2.2. CAVEOLIN-2

3.2.2.1. STRUCTURE

Cav-2 is made of 162 amino acids and is 58% similar and 38 % identical to Cav-1 (80-82). Interestingly, although Cav-2 is very divergent from Cav-1, it is co-expressed with it and is incapable of independent existence. Thus, its tissue distribution is virtually identical to Cav-1. Although Cav-2 has the same domains as Cav-1, it is incapable of forming caveolae on its own (83). However, it forms hetero oligomers with Cav-1(84)

3.2.2.2. FUNCTIONS

Since it is incapable of independent existence, the physiological role of Cav-2 was not discerned until the generation of selective Cav-2 knock out mice (83). These mice exhibit marked pulmonary pathology characterized by interstitial lung disease (83-85) indicating the need for cav-2 in maintaining normal pulmonary structure and function.

3.2.3. CAVEOLIN-3

3.2.3.1. STRUCTURE

Cav-3 is made up of 159 amino acids and is 85% similar and 65% identical to Cav-1. (80 – 82) As discussed previously, Cav-3 expression is limited to the skeletal heart muscle and the smooth muscle (which has all three kinds of caveolin proteins). (80-82)

3.2.3.2. FUNCTIONS

1) Similar to caveolae formed by Cav-1, Cav-3 caveolae also have been found to be enriched in a number of cell signalling molecules. (81, 86 - 91)

2) Recently, studies have shown that Cav-3 probably plays a central role in myocyte differentiation and muscle development. (92)

3) Cav-3 is a part of the dystrophin - glycoprotein complex located on the sarcolemma. (93 -95) Cav-3 is thought to compete with dystrophin to bind with β-dystroglycan to be a part of the dystrophin – glycoprotein complex. Hence, over expression of Cav-3 leads to decreased dystrophin in the complex causing a Duchenne-like muscular dystrophy phenotype. (96 - 99)

4) Loss of Cav-3 has been implicated in other forms of muscular dystrophies including Limb-girdle muscular dystrophy, (100, 101) Rippling muscle disease. (102)

3.2.4. CAV-1 IN TUMOR SUPPRESSION

Numerous experiments have shown that decreasing the levels of Cav-1 leads to malignant transformation of cells. Also, increasing the levels of Cav-1 in these cells restores normal morphology. (103 - 106) These findings suggest that cav-1 has tumor suppressor activity. Cav-1 and Cav-2 have been shown to map to D7S522 locus on 7q31.1. Loss of heterozygosity of this region is observed in a number of human tumors. (107 - 110) Cav-1 is proposed to be the putative tumor suppressor gene of this locus. Significantly, Cav-1 has been shown to be mutated in some human tumors. In a study, 16% of breast cancer samples analyzed were found to have mutated forms of Cav-1. (111) Another study showed mutations in a significant portion of oral squamous cell carcinoma cases (112) providing further evidence that cav-1 is a tumor suppressor gene. Many oncogenes such as c-Myc, Bcr-Abl, HPV E6 have been shown to decrease Cav-1 levels (113 - 118) whereas the tumor suppressor gene p53 was shown to stimulate Cav-1 transcription (118, 119). These findings suggest that Cav-1 probably acts synergistically with other tumor suppressor genes such as p53 and its down regulation is necessary for malignant transformation of cells by oncogenes. Cav-1 knockout mice have yielded valuable information on this gene's function as a tumor suppressor gene. Although these mice do not form spontaneous tumors, they show dramatically increased susceptibility to carcinogens. (120) When these mice where exposed to 7, 12 dimethyl benzanthracene, they showed hyperplastic and malignant changes in the skin. They showed a 10 fold increase in tumor formation and a 15 fold increase in tumor multiplicity. (120) These mice also develop mammary epithelial hyperplasia and dysplasia (121, 122). Cav-1 has been shown to have an inhibitory effect on mammary dysplasia, transformation and metastasis of breast cancer (123, 124). These and other studies provide very strong evidence for the tumor suppressor activity of cav-1.

3.2.4.1. MECHANISM OF TUMOR SUPPRESSION

Although numerous studies provide evidence for the tumor suppressor function of Cav-1, relatively few studies have looked at the mechanism. As discussed above, Cav-1 is a scaffolding protein and helps in anchoring many membrane proteins including receptors and cell signaling pathway components. Cav-1 also has a general inhibitory effect on these pathways.

These pathways include those regulating proto oncogenes – for example, Cav-1 has been shown to inhibit the Ras- p42/44 mitogen activated protein kinase pathway. This pathway, when activated promotes cell cycle progress and cell proliferation.(125 - 128) Proto-oncogenes by definition, are genes that positively regulate cell growth and proliferation, and when mutated cause unregulated proliferation and malignant transformation. (129) Thus, inhibition of these genes causes tumor suppression. Moreover, inhibition of these genes causes inhibition of cell growth and proliferation. (129) Hence, Cav-1 can be thought to inhibit cell growth and cause cell cycle arrest. In fact, Galbiati et. al have shown that Cav-1 is important in cell cycle regulation. Transient transfection of Cav-1 in NIH 3T3 cells caused arrest in the G_0/G_1 phase and a significant decrease in the number of cells in the S phase of the cell cycle. Moreover, down regulation of Cav-1 levels in these cells had the opposite effect. Also, on induction of cell cycle arrest by growing the cells in serum free medium, Cav-1 was up regulated. Addition of serum to the medium resulted in reduction of Cav-1 levels. Similar results were obtained with primary cultures of embryonic fibroblasts from transgenic mice recombinantly expressing caveolin. These experiments conclusively prove that Cav-1 inhibits cell proliferation and cell growth. (130)

Most cells at the end of their proliferative life span reach a state of cellular senescence induced by external (UV radiation, drugs etc.) or internal (free radicals produced due to

physiological processes) stress. The primary features of cell senescence include increased levels of proteins having an inhibitory effect on cell cycle and proliferation (causing cell cycle arrest and inhibition of proliferation), distinct changes in morphology (cells become large and flat), and an increase in acid β-galactosidase activity. (131 – 133) Since senescence inhibits cell growth and proliferation, induction of senescence has been proposed to be an important tumor suppressor mechanism (119, 131, 134-137). Cells with malignant potential that escape cellular senescence and continue unregulated proliferation are thought to cause tumors.

Since it was already shown that Cav-1 inhibits cell proliferation and growth (a key feature of cell senescence), Galbiati et.al next looked into whether Cav-1 could in fact cause cell senescence. If Cav-1 does indeed cause cell senescence, then i) cells over expressing Cav-1 should show premature senescence due to the effects of increased levels of Cav-1 and ii) senescent cells should show increased levels of Cav-1. They found that mouse embryonic fibroblasts from transgenic mice constitutively expressing high levels of Cav-1 underwent premature senescence exhibiting the morphological and physiological features of a typical senescent cell. To show that senescent cells express high levels of Cav-1, they induced senescence in NIH 3T3 cells by subjecting them to stresses such as UV radiation and H_2O_2 and examined the levels of Cav-1. Cav-1 was increased in senescent cells (i.e. cells exposed to UV radiation or H_2O_2) as compared to the control cells showing that Cav-1 is up regulated in stress induced premature senescence (SIPS). (138) Cells expressing constitutively low levels of Cav-1 seemed resistant to induction of premature senescence. Interestingly, the same authors had previously shown that cells expressing constitutively low levels of Cav-1 showed malignant features (126). Hence, these authors provide strong evidence that Cav-1 acts as a tumor suppressor gene by promoting stress induced premature senescence.

However, little is known about the exact mechanisms involved in up regulation of Cav-1 during SIPS. This study aims to remove this deficiency by identifying the molecular mechanisms of this phenomenon.

3.3. EUKARYOTIC GENE REGULATION

Each eukaryotic cell has thousands of proteins which are not required at all times. Most proteins are required at a specific stage of the life of the cell. Also, different tissues of the body have different functions mainly determined by the types and/or levels of proteins they express. Hence, it is essential that gene expression be controlled. Gene expression may be controlled at the transcription, RNA processing and/or translational levels. Protein levels and activity may also be controlled by post translational mechanisms. However, the most important control of gene expression is at the transcriptional level. A typical eukaryotic gene has i)exons which code for the protein, ii)intervening sequences called introns which are removed from mRNA before translation, iii) Distant sequences called enhancers and silencers that modulate the promoter region and iv) a regulatory promoter region that control transcription. These promoter regions are often thousands of base pairs long. The promoter region typically consists of a) a basal or core promoter sequence near the transcription start site that initiates transcription and b) short, upstream promoter sequences where most activity resides. At these sequences, trans-acting proteins called transcription factors, bind. This binding then regulates the protein expression. (129) In the present study, we aim to elucidate the promoter sequences in Cav-1 promoter region important in up regulation of this gene during SIPS and the transcription factors binding to these sequences as a part of this response.

3.4. MAPK SIGNALING CASCADE

Cells have numerous signaling pathways to convey signals from one part of the cell to another or from the extracellular environment to the interior of the cell. These pathways are complex and are mediated by a network of lipids and/or proteins. One such set of pathways that have been shown to be important in transmission of extra cellular signals to regulate cell growth and cell cycle is the Mitogen-Activated Protein Kinase (MAPK) cascade. Five functionally distinct groups of MAPK have been identified so far:

Extra cellular signal-regulated kinases (ERKs) 1, 2 (ERK 1 / 2), c-Jun amino-terminal kinases 1, 2, 3 (JNKs 1, 2, 3), p38 isoforms, ERKs 3,4 and ERK 5. (139)

Of these, ERKs 1, 2, JNKs 1, 2, 3 and p38 cascades have been extensively studied.

These cascades consist of a series of protein kinases that sequentially activate the next member of the cascade by phosphorylation. The general scheme is shown in figure 4.

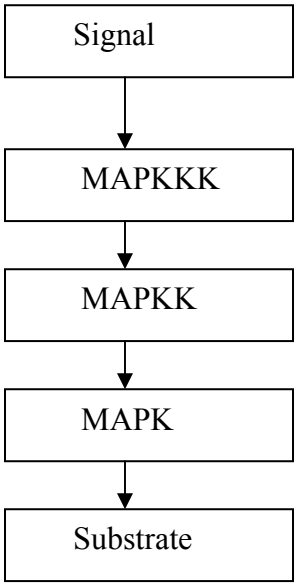

Figure 4

General scheme of MAPK cascade (129)

ERKs 1, 2 have been shown to be activated by growth factors (140) and to primarily modulate cell proliferation (141). In fact, Ras, along with the MAPKKK, Raf which it activates has been shown to be mutated in a large proportion of human cancers (142 - 144) This pathway is specifically inhibited by PD98059 (145)

The p38 module is activated during stress (including oxidative stress) and inflammation. Upon activation, this pathway regulates the expression and activity of many substrates. These substrates among others include transcription factors. (146) This pathway is preferentially inhibited by the compound SB203580 (146)

Although the JNK pathway is also stimulated during stress and inflammation, it is not inhibited by SB203580. (147) Hence, this compound is useful in determining which of these pathways are activated under a particular condition.

4. METHODS

4.1. CELL CULTURE

NIH 3T3 murine embryonic fibroblasts were used in all the cell culture experiments (American Tissue Culture Collection, Manassas,VA) They were cultured in in Dulbecco's Minimum Essential Medium (DMEM) supplemented with

10% donor bovine calf serum (DBCS, Invitrogen, California) and 1% penicillin-streptomycin (Gibco/Invitrogen, California) in a humidified atmosphere of 5% CO_2 at 37°C.

4.2. CONSTRUCTION OF PTA-LUC – CAV-1 PROMOTER PLASMIDS

INTRODUCTION: pTA-Luc is a 4.8 kb vector containing the firefly (*Photinus pyralis*) luciferase gene which is popularly used as a reporter gene in analyzing promoter sequences (148). The promoter sequence under study is introduced immediately upstream to the luciferase gene such that this gene is under the influence of the promoter sequence. The activity of this promoter sequence can thus be determined by measuring the luciferase gene levels. Upstream to the luciferase gene is the TATA box (removed during the preparation of the pTA-Luc-Cav-1 promoter constructs) from the herpes simplex thymidine kinase promoter. In addition, this vector also has an origin of replication (pUC) and an ampicillin resistance gene. The ampicillin gene is useful for selection and propagation of the desired plasmid construct in bacteria such as *E.coli.* Figure 5 shows the various components of the pTA-Luc vector.

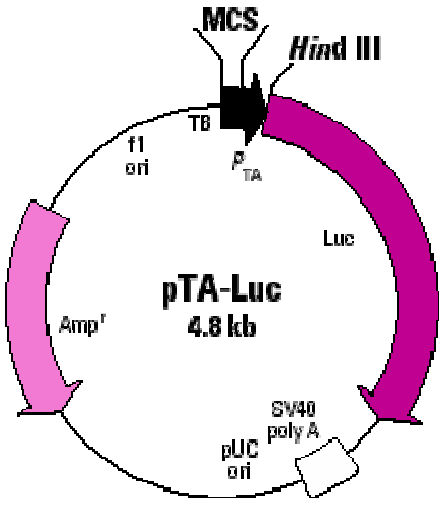

Figure 5

pTA-Luc Vector (pTA-Luc Vector Information, BD Biosciences)

PROCEDURE: The pTA-Luc vector contains the Kpn1 restriction enzyme site at position 6 and an Nco1 restriction site at position 140. These sites are unique to these positions and do not occur elsewhere in the vector. The pTA-Luc vector was digested with Nco1 and Kpn1 restriction enzymes (Roche, Indiana). The vector was first digested with either of the enzymes. The vector thus digested was then precipitated with ethanol and subjected to digestion by the second enzyme. The double digested pTA-Luc vector was then purified by gel extraction and its concentration was determined.

CAV-1 PROMOTER SEQUENCES: The part of the promoter sequence extending from -1 to 1296 of the murine Cav-1 gene was analyzed in this study. This sequence was previously described by Lisanti et al (149) and is shown in figure 6.

1 cttatcagcc gggacccagg ttactggtct tttctcagtt tccattaagc cctgggatt

61 ctcttcatcc ttctcatgag cccgtgctcc tgctttcttg gaaacaattt taaaaatgta

121 ctctctgtcc tttccagtgt gcattgggag tgtttgcata ggaatgctta ggtaaaaatt

181 tcctttcatc cttgaggtgg tggggagtaa actgcttagt tactgtctct aattagttac

241 tctttccttt agtaaaaagc aactatagtt tatcttattt ttccttacaa aagacaaag

301 gaggctggcc agcttttgaa actgatattt tagttgttaa cttaaaaaga aaaactactt

361 tgtcaaaaaa taaaacaaac aaacaaacaa acaaaaaaca ttctctcaca gtcaagcaca

421 tattcctgcg cttggatcct ctatggcaga ggatgtagtt cagtggtaga gtgtttaatc

481 tccaggcaga atacacaaag ccctgggttc gaactcgtcg atcttgagcc aagaaaaaaa

541 aaaaaacaaa aaaaacaaaa acaaaaacaa aaaactctct agtaacaagc ttgaacctct

601 aagtaaaatt ttctgcagtt gaaaatactc aaagtgctta tggttactgt ataacctgct

661 ggtagggatt tcattttctt tattgcagag gcgcccacac agattgcttc gttgtgttta

721 tatttaaaaa aaaaaaaaaa tgttccctca ggttcccagc catctcgctt ctatatcttt

781 ctctgtgaaa caaggagaca gatcagttct acgtgggcga attgggaggg gaggcagctt

841 aggacagggc agaattcttt ctgcagagcc ggatgcccat actgggcatc tctgcagact

901 cttgggctcc ctccacccct gctgagatga tgcactggga aaacacgcgc tctcccctgg

961 atgcctctct gtaggtttat agctgggaaa acgttgcctc gagtctaaaa tatctgccca

1021 aactgcctag ctctgatgaa ggctttctca caggctctca gctccccgcc ggcactcccc

1081 gccctctgct gccagaacct tggggatgtg cctagacccg gcgcagagca cgtcctagcc

1141 aactgagagc agaacaaacc cctggcgaac agccaagagg ctccctccca gccaccgccc

1201 cccgccagcg cctttccccc ctctatacaa tacaagatct tccttcctca gttctcttaa

1261 atcacagccc agggaaacct cctcagagcc tgcagccagc cacgcgccag c

Figure 6

Murine Cav-1 promoter sequence (-1311/-1) (Ref. Lisanti et. al, 149)

Successively truncated segments of the above DNA sequence were prepared by PCR using a previously described 3kb+Intron Cav-1 sequence as template. During this PCR, restriction sites for Nco1 and Kpn1 were also introduced at the 3' and 5' ends respectively. Primers were designed with care and were ordered from Sigma Genosys (Texas). The truncated segments and the primers used in their construction are shown in Table 2. The constructs are shown in Figure 7.

Table 2

Oligos used in the preparation of pTA-Luc –Cav-1 promoter constructs.

Construct	**Length (bp)**	**Forward Primer (5'-3')**
Cav-1 1296/-1	1296	ggccgg**ggtac**ccccaggttactggtcttttct
Cav-1 800/-1	800	ggccgg**ggtac**ctcgtcgatcttgagccaaga
Cav-1 372/-1	372	ggccgg**ggtac**aacacgcgctctcccctggat
Cav-1 222/-1	222	ggccgg**ggtac**ccagaaccttggggatgtgcc
Cav-1 150/-1	150	ggccgg**ggtac**ccctggcgaacagccaagaggc
Cav-1 91/-1	91	ggccgg**ggtac**ccctctatacaatacaagatct

Note: i) For all the constructs, the reverse primer (3'-5') was identical – ggccgg**ccatgg**gctggcgcgtggctggctgca. ii) The sequences in bold are the restriction enzyme sites for Nco1 (cctagg) and Kpn1 (ggctac) introduced at the 3' and 5 ends of the constructs respectively

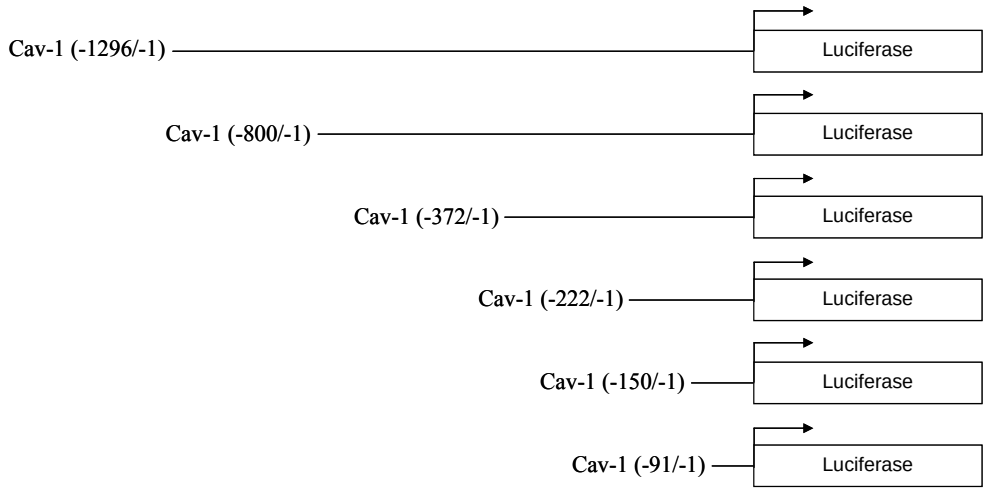

Figure 7

Caveolin promoter – Luciferase reporter constructs

pTa -Luc vector containing the firefly luciferase reporter gene was subjected to digestion by Kpn1 and Nco1 restriction enzymes. 1296 bp and successively shorter fragments (800 bp, 372 bp, 222 bp, 150 bp, 91 bp) of the murine caveolin-1 gene were amplified by PCR. Restriction sites for Nco1, Kpn1 restriction enzymes were introduced during this PCR. They were then subjected to digestion by Kpn1 and Nco1 and ligated to the previously digested pTA- Luc vector to create Cav-1 Pr.1296, Cav-1 Pr. 800, Cav-1 Pr.372, Cav-1 Pr. 222, Cav-1 Pr.150, Cav-1 Pr.91 promoter – reporter constructs respectively.

CONSTRUCTION OF PROBES A, B, A MUT, B MUT: Probes A and B contained the minimal promoter regions of the murine Cav-1 promoter region essential for up regulation of Cav-1 during SIPS. Probe A (having region -240/-218) encompassed the proximal Sp1 consensus sequence identified in the Cav-1 372/-1 construct whereas Probe B (having region -122/-100) had the distal Sp1 consensus sequence identified in Cav-1 150/-1 construct. Probes A Mut and B Mut contained the corresponding regions with mutations in the critical consensus Sp1

binding site sequences. The 3' primer was designed containing an Nco1 restriction site. The 5' primers were designed containing the Kpn1 restriction site as above. However, additionally, the 5' primers also had the consensus Sp1 binding sites. Moreover, these primers were designed such that the region on pTA-Luc between Kpn1 (position 6) and Nco1 (position 140) could be amplified when PCR was carried out with pTA-Luc as a template. The fragments thus obtained were gel purified and were ligated into the double digested pTA-Luc vector as described above. The primers used are as follows:

Table 3

Oligos used in the preparation of Probes A, B, A Mut, B Mut. Note: For all the constructs, the reverse primer (3'-5') was identical – ggccgg**ccatg**gtggctttaccaacagtaccgg ii) The sequences in bold are the restriction enzyme sites for Nco1 (cctagg) and Kpn1 (ggctac) introduced at the 3' and 5 ends of the constructs respectively iii) The sequences in bold and underlined are the consensus Sp1 sites (Probes A and B) or the consensus Sp1 sites with mutations (Probes A Mut and Probe B Mut)

Construct	**Forward Primer (5'-3')**
Probe A	ggccgg**ggtac**cggcact**<u>ccccgccc</u>**ctctgctgccagctctacgcgtgctagcc
Probe A Mut	ggccgg**ggtac**cggcact**<u>tttttttcc</u>**tctgctgccagctctacgcgtgctagcc
Probe B	ggccgg**ggtac**ccagcca**<u>ccgcccccccgcc</u>**agcgcagctcttacggcgtgctagcc
Probe B Mut	ggccgg**ggtac**ccagcca**<u>cctttttttttcc</u>**agcgcagctcttacggcgtgctagcc

RESTRICTION ENZYME DIGESTION: Nco1 and Kpn1 restriction enzymes were obtained from Roche Pharmaceuticals. The digestions were carried out with either of the enzymes in a solution containing 1X enzyme buffer and 100 µg/ml bovine serum albumin (for Kpn1 only). The final solution containing the DNA, restriction enzyme, sterile water, buffer and bovine serum albumin was resuspended several times and left in a water bath at 37 C overnight.

ETHANOL PRECIPITATION: The DNA digested by one of the two restriction enzymes was then isolated and purified from the solution by ethanol precipitation. Briefly, the volume of the mixture was brought to 200 µl. 500 µl of 100% ethanol and 20 µl of sodium acetate (pH 5.4) were added and the contents mixed by inverting the tube repeatedly. The tube was then placed in dry ice for 20 minutes before centrifuging for 10 minutes at 4 C at 16,000 x g. The supernatant was then discarded and 200 µl of 70 % ethanol was added. The tube was then centrifuged at 4 C for 5 minutes at 16,000 x g. The supernatant was again discarded and the traces of ethanol left in the tube were allowed to evaporate by letting the tube stand for 2-3 minutes. The DNA was then resuspended in sterile water.

The digestion with the second enzyme was subsequently carried out as already described.

POLYMERASE CHAIN REACTION (PCR): PCR was carried out in a PTC – 100 system (MJ Research Inc). The reactions were usually carried out in a final volume of 50 µl containing 5 µl of 10 X Buffer (Roche Pharmaceuticals, Indiana) 4 µl dNTP, 5 µl 5' primer, 5 µl 3' primer, 0.5 µl Taq polymerase, 100 µg template DNA and sterile, nuclease free water. Extreme precaution was taken to prevent any contamination of the PCR reactants and products. An initial denaturation step was carried out at 94 C for 5 minutes followed by 30 cycles of denaturation at 94 C (30 seconds) annealing at the appropriate temperature depending on the relative A,T,G,C content of the primers (45 seconds) and extension at 72 (1 ½ minutes). After a final extension

step at 72 C for 7 minutes, the reaction was stopped by allowing the PCR products to cool to 4C. The PCR products were then purified and their relative quality and concentration of the PCR products was determined by running a 0.8 % agarose gel.

PCR PURIFICATION: A Qiaquick PCR purification kit (Cat. No. 28104) was obtained from Qiagen Inc. (California) and the DNA in the PCR mixture (containing primers, enzyme, salts etc) was separated according to the manufacturer's instructions.

TAE AGAROSE GEL ELECTROPHORESIS: This was carried out to i) separate the various DNA components and obtain the restricted enzyme digested product by gel purification, ii) determine the concentration of this digested product and iii) determine the size of the digested fragment by comparing it to DNA marker ladder. Briefly, 0.8 gm of DNA grade agarose (Fisher Biotech) and 2ml of 50X TAE were mixed in 100 ml distilled water and placed in a microwave for 1 ½ minutes for the agarose to dissolve completely. 3 µl of ethidium bromide solution (Fisher Biotech) (for fluorescence under UV light) was added to the agarose solution before pouring it into a cast to form a gel. The gel was allowed to solidify for 20 minutes before loading the samples. Meanwhile, the running buffer was prepared by diluting the 50X TAE in distilled water to produce a final concentration of 1X TAE. 1 µl 6X TAE with bromophenol blue was added to 5 µl of sample and resuspended before loading on the gel. The gel was allowed to run for an appropriate length of time and the gel was examined under UV light.

GEL PURIFICATION: This technique was used to separate the digested product from the undigested product and other unwanted DNA material. Gel electrophoresis was carried out as described above and examined under UV light. The desired digested fragment was cut out and purified from the gel using a Gel Extraction Kit (Cat. No 28704) obtained from Qiagen Inc. (California).

DETERMINATION OF CONCENTRATION OF DNA: Gel electrophoresis was carried out as described above and the concentration of DNA was determined by the strength of fluorescence of the bands of DNA when examined under UV light. The digested pTA and Cav-1 promoter fragments thus obtained were then ligated.

LIGATION: Ligation was carried out in sterile Eppendorf tubes at a final volume of 10µl. The final mixture contained the digested 40 ng pTA-Luc, Cav-1 promoter sequence in varying concentrations, 1µl of 10X ligation buffer (Roche Pharmaceuticals, Indiana) 1µl of ligase (Roche Pharmaceuticals, Indiana) and sterile water. The eppendorf tubes were left overnight at room temperature after resuspending several times. The ligated products were then used to transform DH5ά *E.coli* bacteria (Invitrogen). An eppendorf tube to which the promoter sequence was not added (i.e. containing only pTA-Luc) was used as a control.

BACTERIAL TRANSFORMATION: 50µl of DH5ά *E. coli* bacteria were added to the eppendorf tube containing the ligation product and mixed by gently tapping the tube. They were then placed in ice for 30 minutes. Heat shock was performed by placing the eppendorf tubes in a water bath at 37 C for 30 seconds. The tubes were then put in ice for 2 minutes before 500 µl of LB (Luria - Bertani) medium was added to them .They were then placed on a shaker at 37 C for 1 hour. The contents of each tube were then plated on to an LB plate with ampicillin. These amplicillin plates were then left at 37 C overnight. The plates were then examined the next day and the plate with the highest number of colonies as compared to the control plate (pTA-Luc without construct) was selected. A single colony from this plate was then cultured in 5ml of LB medium with amplicillin overnight and a mini prep was performed on the broth to obtain the plasmid DNA.

EXTRACTION OF DNA FROM BACTERIAL CULTURE (MINIPREP): A Qiagen Miniprep kit was obtained from Qiagen Inc. (California) and the DNA was extracted according to manufacturer's instructions. Glycerol stock of this culture was also prepared by adding 500 µl of the broth added to 500µl of a solution containing 60% LB and 40% glycerol The glycerol stock was stored at -80 C and used for subsequent cultures.

The plasmid DNA thus obtained was subjected to digestion with Nco1 and Kpn1 restriction enzymes and the products were subjected to gel electrophoresis to confirm the size of the fragments.

EXTRACTION OF DNA FROM BACTERIAL CULTURE (MAXIPREP): This technique was used to extract the plasmid DNA from the bacterial culture on a large scale. About 100 µl of the glycerol stock prepared previously was cultured in 5 ml LB with ampicillin for 7-8 hours at 37 C with vigorous shaking. This broth was then added to 500 ml of LB medium with ampicillin and grown overnight at 37 C with vigorous shaking. A Qiagen Maxiprep kit was obtained from Qiagen Inc. (California) and the plasmid DNA was extracted according to manufacturer's instructions. The concentration of the DNA thus obtained was determined using a UV spectrophotometer (Ultrospec 2100 pro).

CONSTRUCTION OF pcaggs-Sp1 cDNA PLASMIDS: Total mRNA was collected from mouse lung cells using Qiagen RNeasy Easy Kit (Qiagen Inc,.California). Reverse Transcriptase PCR (RT-PCR) was done using a kit obtained from Qiagen to produce cDNA from the mRNA extracted previously. Sp1 cDNA in the cDNA mixture was then selectively amplified by PCR using primers specific for Sp1 gene: ccggcc**gaattc**atgagcgaccaagatcactcc(5'), ccggcc**agatct**ttaattcagatcttcctcgctgatcagtttctgttcgccgccgaaaccattgccactgatatt (3').The sequences shown in bold are restriction sites that were introduced for EcoRI (5') and BglII (3'). The

construct was then made as described previously. Briefly, pcaggs vector and Sp1 cDNA were digested with the EcoRI and BglII restriction enzymes and ligated. Transformation of bacteria, miniprep, maxiprep were done to obtain the pcaggs-Sp1 construct having the Sp1 gene under the control of a constitutively active chicken actin promoter.

4.3. LUCIFERASE ASSAY

CELL CULTURE: NIH 3T3 cells were cultured in 6-well plates (Corning Inc, NY) using 1.5 ml of supplemented DMEM medium at 37 C and 5 % CO_2 in an incubator (Forma Scientific Inc) such that they were approximately 25-35 % confluent on the day of transfection.

TRANSFECTION: On the day of transfection, the medium was replaced with fresh medium 4-5 hours before transfection to increase the efficiency. Transfection was carried out by the modified calcium phosphate method. A vector containing β - galactosidase (β-gal) gene under the control of a CMV derived constitutive promoter was used as to control for varying numbers of cells and efficiency of transfection between different dishes. A mixture of 2.5 µg of the pTA-Luc Cav-1 promoter construct and 1.5 µg of β-gal DNA was prepared in each tube and the final volume in each tube was brought to 65µl with 2X calcium chloride solution ($Cacl_2$ 250 mM, Tris 1mM, EDTA 0.1 mM). In the experiments with pcaggs-Sp1 constructs, 2 µg each of the Cav-1 promoter construct and Sp1 construct, 1 µg of β-gal DNA were used. 65 µl of this mixture was then added to an eppendorf tube containing 2X HeBS (Nacl 280mM, Hepes 50nM, Na_2HPO_4 7 H_2O 1.5 mM, pH 7.07) in a drop wise manner over 30 seconds thus bringing the final volume to 130 µl. The tube was then vortexed for 15 seconds. This mixture was then allowed to incubate for 20-25 minutes. It was then added to the 6 well plates in a drop wise fashion taking 1 minute per dish. The dishes were then swirled a few times and placed at 37 C for 15 -16 hours. The cells

were then washed twice with PBS and the medium was replaced. The medium that was replaced contained150 µl/ml H_2O_2 to induce senescence when necessary.

INDUCTION OF OXIDATIVE STRESS: As discussed elsewhere, treatment with H_2O_2 has been shown to be a good model to study stress induced premature senescence. In the present study, the transfected cells were treated with 150 µM H_2O_2 for 2 hours. The dishes were then washed twice with DMEM medium at the end of two hours and added with 1.5 ml supplemented DMEM. To inhibit the p38 MAPK, SB 203580 (15 mM in DMSO) was added to the dishes to reach a final dilution of 10 µM.

LUCIFERASE ASSAY PROTOCOL: The cells were collected 48 hours (when Sp1 cDNA was co-transfected) or 72 hours after transfection (when cells were subjected to H_2O_2 treatment to induce oxidative stress). The collection of cells and all subsequent steps were carried out on ice. Cells were then washed twice with PBS at 4 C and 500 µl of extraction buffer (1% w/v Triton X-100, 1 mM DTT in GME buffer) was added and the dishes placed on a shaker at 4 C for 30 minutes. Later, 100 µl of cell extract from each dish was added to i) BD Monolight luminometer cuvettes (BD Monosciences) containing 300 µl of ATP mix (100 mM potassium phosphate, 100mM DTT, 200 mM ATP in GME buffer) for luciferase assay and ii)Eppendorf tubes containing 600 µl of Z buffer, pH 7 (100mM sodium bi phosphate, 10mM potassium chloride, 1mM magnesium sulphate, 50 mM, β- mercaptoethanol) for β-galactosidase assay. The luminometer cuvettes were then read using a luminometer that injected 100 µl of luciferin substrate **(**Molecular Probes, Oregon) solution per tube (1mM luciferin substrate, 50 mM DTT).

β-galactosidase assay: 40 µl of chlorophenol red-β-galactopyranoside solution (CPRG, Boehringer Mannheim) was added to the eppendorf tubes with 100 µl cell extract and 600 µl of Z buffer and the reaction was allowed to proceed for 5 minutes. The reaction was then stooped

using 400 µl of 1 M sodium carbonate. The absorbance of each sample was read at 574 nm using a UV spectrophotometer.

The readings from the luciferase assay were then normalized with those from the β-galactosidase assay to account for differences in cell number and/or transfection efficiency.

All the transfections were done in duplicate and repeated twice. Also, in each experiment, two samples were collected from each dish.

4.4. PREPARATION OF NUCLEAR EXTRACTS

NIH 3T3 cells were cultured in p100 dishes (Corning Inc., NY) as before. Later, they were then treated with H_2O_2 as before to induce oxidative stress. 48 hours later, nuclear extracts were prepared using a nuclear extraction kit (Cat. No.78833) obtained from Pierce Biotechnology (Illinois) and the extraction was done according to the manufacturer's instructions. As a control, nuclear extracts were also prepared from p100 dishes that were not treated with H_2O_2. Care was taken to ensure that both groups of dishes were of similar confluency.

4.5. ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

A non radioactive biotin based chemilumniscent EMSA technique was used in this study.

3' end biotin - labeled single stranded oligos were designed containing the consensus Sp1 sequences. As competitors, non labeled oligos with identical sequences were designed. The oligos were ordered from Sigma Genosys and were as follows:

Table 4

Single stranded oligos that were used in the preparation of double stranded probes for EMSA

Probe	5'-3' sequence
Probe A1-B	ggcact**ccccgccc**ctctgctgcc [3BTN]
Probe A2-B	ggcagcagagggcggggagtgcc [3BTN]
Probe B1-B	cagcca**ccgccccccgcc**agcgc [3BTN]
Probe B2-B	gcgctggcgggggggcggtggctg [3BTN]
Probe A1	ggcact**ccccgccc**ctctgctgcc
Probe A2	ggcagcagagggcggggagtgcc
Probe B1	cagcca**ccgccccccgcc**agcgc
Probe B2	gcgctggcgggggggcggtggctg

Annealing: The oligos were re suspended in an eppendorf with Annealing Buffer (10mM Tris, 0.5 mM EDTA, 0.5mM trisodium phosphate, 1M sodium chloride in sterile water) The oligos were serially diluted from the stock to a final dilution of 50 fmol/µl (biotin labeled oligos) or 10 picomol/µl (unlabeled DNA). Equal volumes of complementary single stranded oligos were resuspended in an eppendorf tube and the tube was placed in a beaker with 500 ml of water at 95 C. The beaker was then allowed to cool slowly to room temperature.

GEL ELECTROPHORESIS: The oligos were then incubated with nuclear extracts from dishes treated with and without H_2O_2 and run on a 5 % non denaturing gel with TBE (Tris, Boric Acid, and EDTA) buffer. The gel was transferred onto a positively charged nylon membrane (Pierce Biotechnology, Illinois) and the membrane was developed using a kit (Pierce Biotechnology, Illinois) according to the provided instructions.

4.6. WESTERN BLOT

3T3 cells were cultured in 100 mm dishes as described above. When they reached 40-50% confluency, the dishes were treated with H_2O_2 as before. When required, 10 μM SB 203580 was added to the medium for 48 hours to inhibit the p38 MAPK cascade. Cells were collected after 48 hours in 1X boiling LB and boiled again for 5 minutes to denature the proteins. Samples were stored at – 20 C until further use. Protein concentration was measured using the Bradford Method. Samples were subjected to SDS-PAGE in a 12.5 % gel. The gel was then transferred overnight onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) at 150 mA. The required regions of the membrane were then cut and blotted in a blocking solution containing 1 gm non fat dry milk (Nestle, Ohio) in 50 ml TBS-T for 2 hours. 0.5 gm bovine serum albumin (Sigma Genosys, Texas) was added to the above solution when the membrane was to be blotted with polyclonal antibodies. The membrane was then washed thrice with TBS-T for five minutes (15 minutes in total) and was blotted with the antibody solution (1:100 for polyclonal anti-phospho p38 MAPK Ab and polyclonal anti p38 MAPK Ab and 1:500 for monoclonal anti-caveolin-1 Ab - diluted in TBS-T) overnight. Both the polyclonal antibodies against p38 were obtained from Cell Signaling Technology (Massachusetts) and the monoclonal antibody against Cav-1 from BD Biosciences (California). The next day, the membrane was again washed as

above and a secondary antibody was added. An anti-mouse antibody (diluted 1:2,500 in blocking solution) and an anti-rabbit antibody (diluted 1:1,000 in blocking solution) were used as secondary antibodies for the monoclonal and polyclonal primary antibodies respectively. Since the secondary antibodies were conjugated to peroxidase, the signal could be detected with a chemilumniscent kit obtained from Pierce Biotechnology (Illinois) according to the provided instructions.

5. RESULTS

Two oxidative stress responsive elements, between bp 91 & bp 151 and bp 222 & 372: Fig 8 compares the luciferase activity of the empty pTA-Luc vector without any construct with that of the pTA-Luc- Cav-1 promoter constructs. The activity seen with the empty pTA-Luc vector on treatment with H_2O_2 is the baseline value due to activity of some unknown sequences in the vector that respond to the treatment. However, this increase in activity of the vector is insignificant compared to the increases seen with the constructs and can be safely ignored. Cav-1 Pr 1296 was activated by oxidative stress. Moreover, Cav-1 Pr 1296, Cav-1 Pr 800 do not show a significant difference in activity on treatment with H_2O_2. Cav-1 Pr 222 shows a 4 fold drop in activity as compared to Cav-1 Pr 372. This suggests that the sequence responsible for at least a part of the Cav-1 promoter activity lies in the region from 222 bp to 372 bp i.e in the region deleted between Cav-1 Pr 372 and Cav-1 Pr 222. Similarly, although Cav-1 Pr 222 and Cav-1 Pr 150 do not show any significant difference in activity, a 5 fold drop is noticed from Cav-1 Pr 150 to Cav-1 Pr 91 suggesting that there is another sequence between bp 91 and bp 150 where a part of the activity of the Cav-1 promoter resides. Although theoretically there could be another sequence downstream of bp 91, it is highly unlikely since the activity of this sequence is not significantly different from the empty vector.

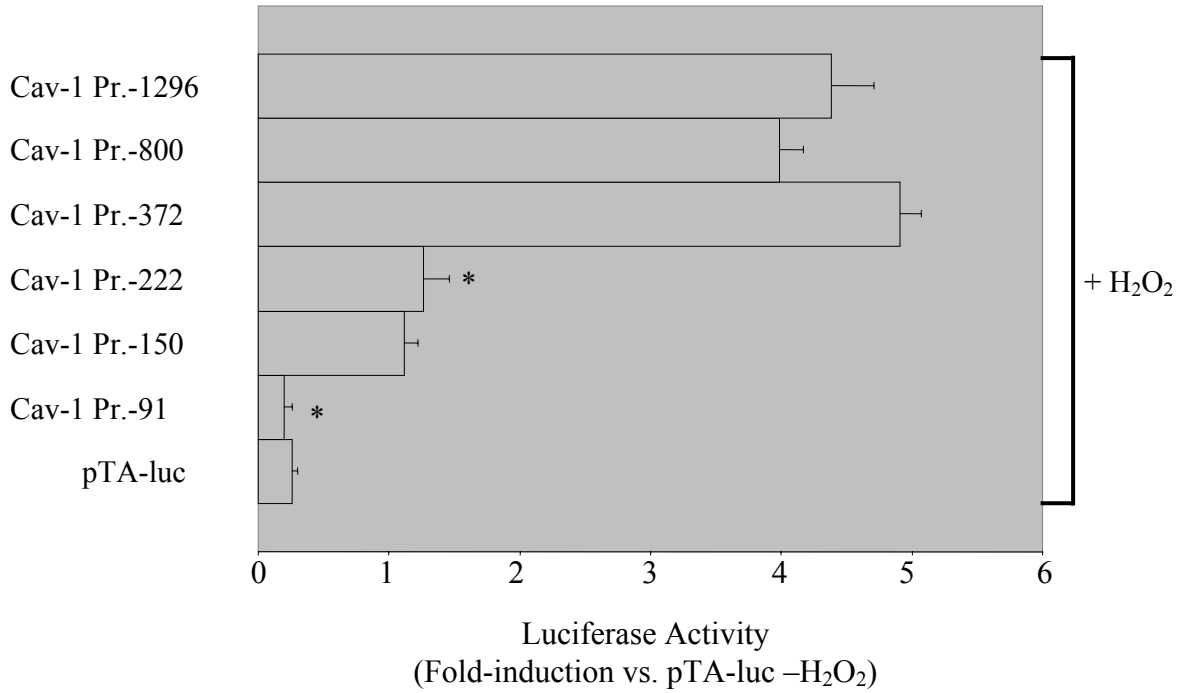

Figure 8

Oxidative stress responsive elements are in regions -91/-150 and -222/-372

NIH 3T3 cells were transiently transfected with each caveolin-1 promoter luciferase reporter construct and were treated with H2O2 for 2 hours. Cells were then lysed and luciferase activity measured. A beta-galactosidase expressing vector was co-transfected as internal control. Note that promoter activity in oxidative stress does not change significantly from Cav-1 Pr.1296 to Cav-1 Pr.372. However, it drops approximately 4 -fold from Cav-1 Pr.372 to Cav-1 Pr.222 and 5 -fold from Cav-1 Pr.150 to Cav-1 Pr.91. This indicates that the elements critical for response to oxidative stress are in the regions deleted between these constructs i.e. -91/-150 and -222/-372. The results are the means ± S.E. of four independent experiments. *Significant reduction in promoter activity ($P < 0.05$).

Segments identified have Sp1 consensus sites: The segments between bp 222 & bp 372 and bp 150 & bp 91 were analyzed**.** Both these segments were found to have one Sp1 consensus site each i.e GC-rich having sequences shown to bind Sp1 transcription factor (161). These two sequences were at -117/-112 and -233/-228. Since Sp-1 was already shown to be important in regulating Cav-1 gene expression under other conditions, (151, 152) these sequences were looked at as the possible oxidative stress response elements.

Sp1 consensus sites respond to H_2O_2 treatment: To demonstrate that these sequences responded to H_2O_2 treatment, Probes A and B pTA-Luc vector containing the Sp1 consensus sequences were constructed as described before. Fig 9 shows that the sequences included in the probes showed some activity that increased manifold upon treatment with H_2O_2. This shows that these sequences are responsive to oxidative stress and are possibly the sites mediating up regulation of Cav-1 in H_2O_2 induced stress.

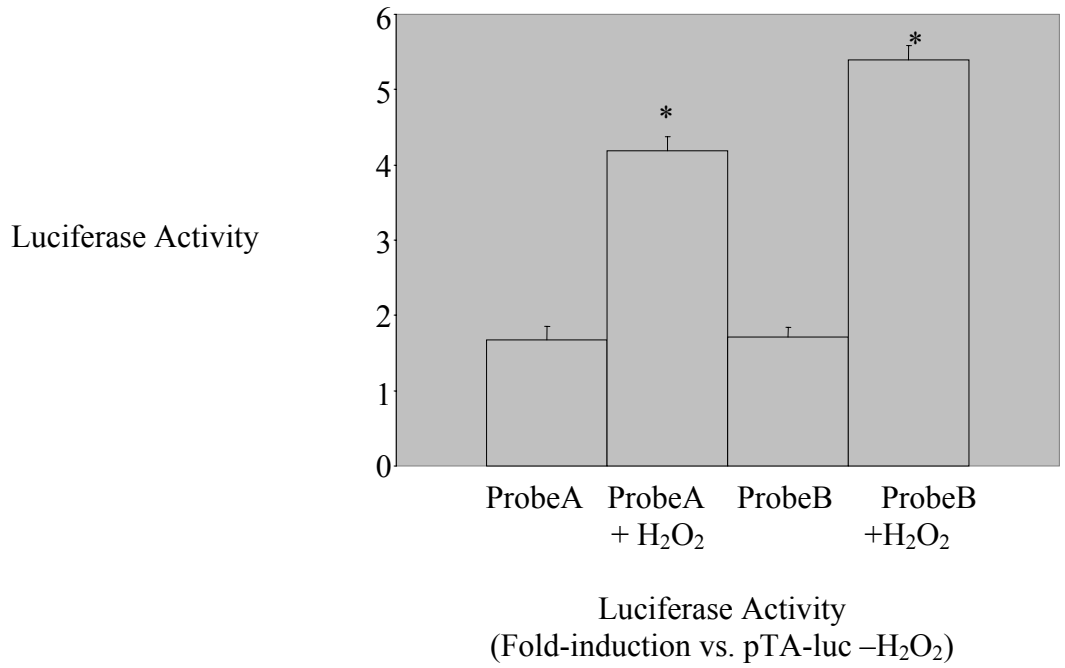

Figure 9

Sp1 consensus sites are responsive to oxidative stress

Transfection and treatment with H_2O_2 was carried out as described previously. Probe A and B showed significant rise in promoter activity with H2O2 as compared to untreated counterparts. This shows that the Sp1 consensus sites are the critical elements in regions -91/-150 and -222/-372 that respond to treatment with H2O2. The results are the means ± S.E. of four independent experiments. *Significant increase in promoter activity due to H202 treatment ($P < 0.05$).

Sp1 binds to Sp1 consensus sites: To show that the increase in activity seen at these sites is due to binding of Sp1 transcription factor, a) An electrophoretic mobility shift assay was done to show that there is transcription factor binding to these sites and this is increased during oxidative stress and b) A luciferase assay to prove that the transcription factor binding to these sites is in fact Sp1 were carried out. For this assay, DNA containing the sequence of interest i.e. transcription factor binding site) is incubated with nuclear extracts containing transcription factors. It is then run in a gel under current along with identical free DNA that has not been incubated with nuclear extract as control. If the DNA sequence does bind to transcription factors, then a transcription factor: DNA complex is formed that migrates slower than free DNA. DNA is usually labeled with radioactive ^{32}P or Biotin to enable visualization of DNA as a band when the gel is transferred onto a membrane and developed. Moreover, if the interaction between the DNA and transcription factor is specific, when an excess of unlabelled DNA identical to the sequence being studied is added, the band is no longer visible since the unlabeled DNA competes with the labeled DNA for the transcription factor. In this study, EMSA was done with biotin (B) labeled DNA sequences containing the consensus sites (Probe A-B, Probe B-B). Free Probes A-B and B-B DNA (lanes marked "No extract"), Probes A-B and B-B incubated with nuclear extracts from cells not treated with H_2O_2 (lanes marked - H_2O_2), incubated with nuclear extracts from cells treated with H_2O_2 (lanes marked + H_2O_2) and Probes incubated with nuclear extracts from cells treated with H_2O_2 along with excess unlabeled probes were subjected to gel electrophoresis. The results are shown in Fig 10. Lanes without any extract show the mobility of free DNA which can be seen at the bottom of the gel, marked as free probe. There is a shift in the lanes incubated with nuclear extracts. This shows that there is binding of a transcription factor to these sites. In these lanes, two bands are seen. Of these, one is non-specific since it is seen in all the lanes including

the lane with excess unlabeled probe and is marked non-specific. The specific band, marked Complex-1 is lost when an excess of the unlabeled probe is added thus proving its specificity. This shows that there is binding of transcription factor(s) to the Sp1 consensus sites. Significantly, this is stronger in the lanes with nuclear extract from cells treated with H_2O_2. This shows that there is increased binding of the transcription factor to the Sp1 consensus sites i.e. increased promoter activity at these regions during H_2O_2 treatment. Fig 9 thus proves that there is increased transcriptional activation at both the Sp1 consensus sites in response to H_2O_2 treatment. However, the nuclear extracts contain numerous other transcription factors apart from Sp1 and theoretically, any of these could be binding to the Sp1 consensus sites. To prove the identity of this transcription factor as Sp1, a luciferase assay was done with Probes A and B and the corresponding mutants (Fig 11). They were co –transfected along with the pcaggs- Sp1 construct. This construct has the Sp1 gene under chicken β - actin promoter which is constitutively active. Thus these cells had very high levels of Sp1 and Probes A and B (or the mutant sequences). There was a dramatic increase in luciferase activity with Probes A and B with Sp1 (probe A + Sp1, Probe B + Sp1) as compared to the probes without Sp1 (Probe A - Sp1, Probe B - Sp1). Importantly this increase is lost with the mutant Probes (Probe A Mut + Sp1, Probe B Mut + Sp1) This shows that there is an interaction between the Sp1 consensus sites and the Sp1 produced by the Sp1 cDNA and that this interaction is sequence specific. This proves that the transcription factor binding to the Sp1 consensus sites is indeed Sp1.

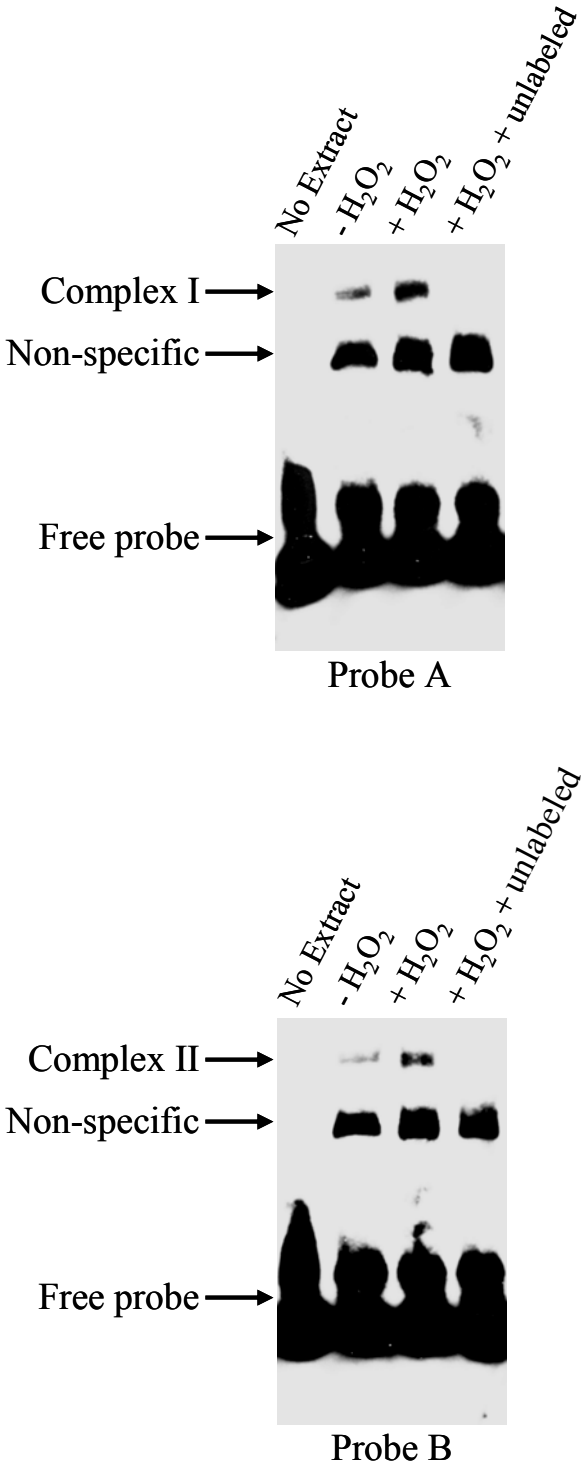

Figure 10

Figure 10 Transcription factors bind to Sp1 consensus sites

Nuclear extracts were prepared from H2O2 treated and untreated cells incubated with biotin labeled Probes A and B and were subjected to gel electrophoresis. Both probes show increased binding to transcription factors with extracts from H2O2 treated cells. This shows that there is increased promoter activity (i.e. up regulation) at these sites in response to oxidative stress. Also no visible band is seen when excess unlabelled probe is added showing that the binding at this site is specific. In contrast, a non-specific band unaffected by unlabelled probe is seen in all lanes with extract. Free probe may also be observed at the bottom of the lanes.

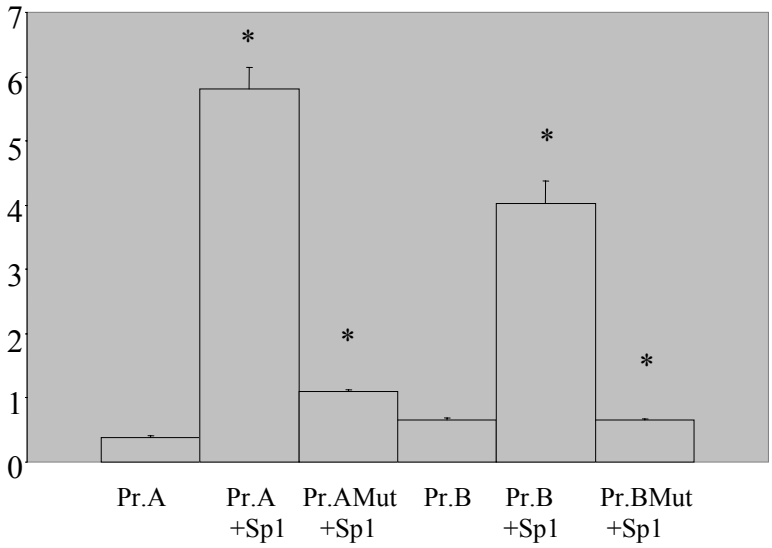

Figure 11

Transcription factor binding to Sp1 consensus sites is Sp1

Gel shift assay (Figure 9) shows increased transcription factor binding to Sp1 consensus sites in response to oxidative stress. To confirm the identity of this transcription factor as Sp1, cells were transfected with Probes (A and B) or mutant Probes (A MUT and B MUT) with or without a vector with Sp1 cDNA under the control of a constitutive promoter and luciferase assay was performed. Sp1 dramatically activates both Probes A and B. In contrast, mutant probes show drastic decrease in activity. This shows that Sp1 binds to the consensus sites and this binding is sequence specific. The results are the means ± S.E. of four independent experiments. *Significant increase in promoter activity due to Sp1 co- trasfection ($P < 0.05$).

Quercetin decreases activity of Sp1 consensus sites: Quercetin is a naturally occurring plant flavinoid shown to have strong antioxidant properties. This antioxidant property could be due to its free radical scavenging ability. Galbiati et. al had shown previously that quercetin prevents the increase in Cav-1 levels following treatment with H_2O_2 and that this is due to prevention of the usual increase in Cav-1 promoter activity. (138) To further study this phenomenon, cells transfected with Probe A or B were treated with H_2O_2 alone or H_2O_2 along with quercetin (Fig 12)**.** As expected, both the probes showed an increase in promoter activity when treated with H_2O_2 (Lanes marked Probe A and Probe B). This activity was abolished when quercetin was added (Lanes marked Probe A/B + quercetin). Note that all the lanes indicate data from H_2O_2 treated cells. This shows that the effect of quercetin on Cav-1 promoter during oxidative stress is at least in part through the Sp1 consensus sites. It can be concluded that quercetin scavenges the free radicals produced from H_2O_2 and prevents the activation of downstream pathways that up regulate Cav-1 expression through the binding of Sp1 to the Sp1 consensus sites.

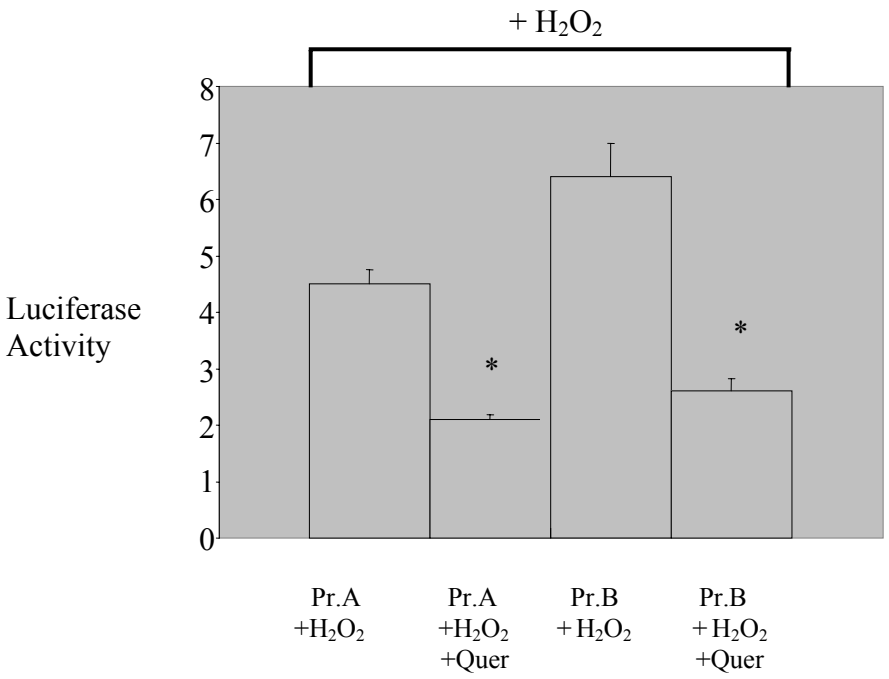

Figure 12

Antioxidants abolish upregulation of Cav-1

Fig 9 shows that the Sp1 consensus sites are the critical elements in regions -91/-150 and -222/-372 responding to H2O2. This response was abolished when quercetin, an anti-oxidant, was added along with H2O2. This can be explained by the fact that anti-oxidants scavenge free radicals produced by H2O2 and reduce stress. This also shows that senescence could be delayed by anti-oxidants. The results are the means ± S.E. of four independent experiments. *Significant reduction in promoter activity due to quercetin ($P < 0.05$).

p38 MAPK mediates oxidant induced up regulation of cav-1: To further elucidate the pathways involved in up regulation of Cav-1 during oxidative stress, we carried out western blots as described above to check for the role of p38 MAPK if any. Figure 13 shows an increase in the level of phosphorylated form of this enzyme (p-p38, upper panel) following oxidative stress (ctl vs + H_2O_2) while the total level (p38, lower panel) remain constant. This suggests that although the total p38 level does not change after oxidative stress, the level of the phosphorylated form increases, i.e. p38 is activated in response to oxidative stress. We then asked whether the p38 MAPK pathway modulates the up regulation of Cav-1 after H2O2 treatment.

We looked at expression of Cav-1 after H_2O_2 treatment in the presence of the p38 MAPK inhibitor, SB 203580**.** Fig 14 shows that the usual increase in level of cav-1 during oxidative stress is lost when SB 203580 is added. Since SB 203580 has been shown to inhibit the activity of phosphorylated p38 MAPK, it can be concluded that inhibition of p38 MAPK activity prevents the usual up regulation of cav-1 during oxidative stress. This shows that the p38 MAPK pathway is vital for the increase in the levels of cav-1 during oxidative stress.

To further show that this increase in cav-1 is in fact due to increased transcriptional activity (i.e. increased promoter activity at the previously identified Sp1 consensus sites), we carried out a luciferase assay as described before. Briefly, 3T3 cells were transfected with Probe A or B and subjected to oxidative stress in the presence or absence of SB 203580. Figure 15 shows a significant reduction in promoter activity at the Sp1 consensus sites in response to oxidative stress in the presence of SB 203580. Thus, figure 15 shows that the mechanism of increase in cav-1 levels by p38 MAPK is at least in part through increased transcription via the previously identified Sp1 consensus sites. In light of the above data, we propose that in response to oxidative stress, p38 MAPK is activated by phosphorylation and probably increases the levels

and/or activity of Sp1 directly or through other downstream elements. Sp1 then causes an increase in transcriptional activity at the Sp1 consensus sites resulting in increased cav-1 protein levels. Cav-1 then causes stress induced premature senescence through as yet unknown mechanisms.

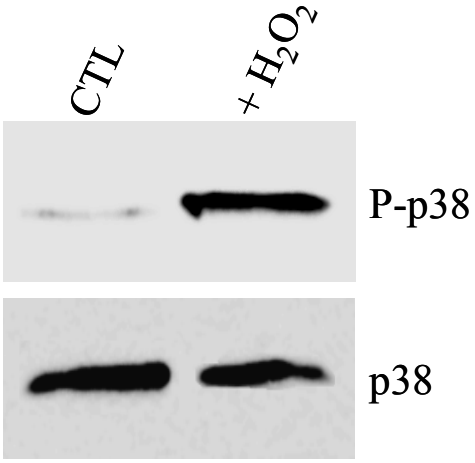

Figure 13

Active p38MAPK increases after oxidative stress

p38 levels at 48 hours in H_2O_2 treated and untreated cells were compared. The total p38 level does not change (lower panel) while the level of the phosphorylated form increases significantly (upper panel) after H_2O_2 treatment suggesting a role for this cascade in increasing Cav-1 levels after oxidative stress

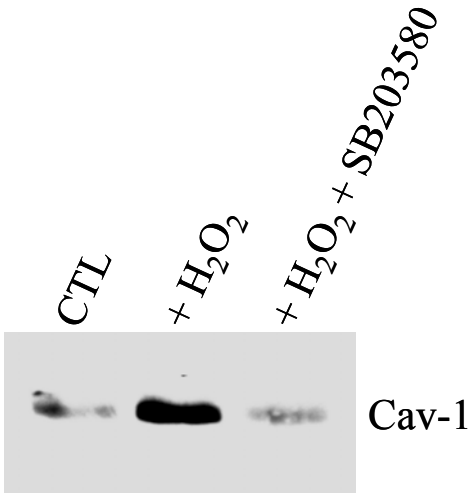

Figure 14

Phospho p38 is necessary for oxidant induced up regulation of Cav-1

The previous figure suggests that p38 MAPK might have a role in the oxidative stress induced increase in Cav-1. To confirm this, Cav-1 levels were compared in H_2O_2 treated cells with and without SB 230580, a known inhibitor of this enzyme. SB 230580 abolished the usual increase in Cav-1 levels following H_2O_2 treatment confirming a central role for p38 MAPK in this process.

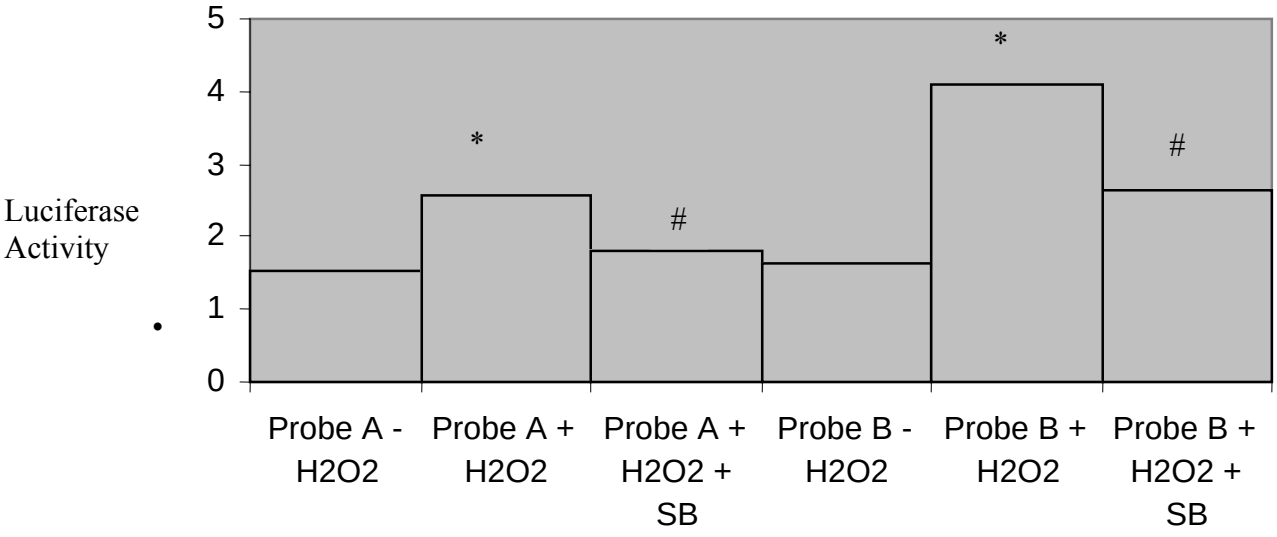

Figure 15

Phospho p38 is necessary for oxidant induced up regulation of Cav-1

To further show that p38 MAPK mediates the H2O2 promoted increase in Cav-1 levels through increased transcriptional activity, a luciferase assay was performed on cells transfected with Probe A or Probe B. These cells were then subjected to H_2O_2 treatment in the presence or absence of SB 203580. A significant drop in promoter activity at the previously identified Sp1 consensus sites is seen in the presence of SB 203580. This shows that p38 MAPK increases Cav-1 levels after oxidative stress through increased transcriptional activity at the two Sp1 consensus sites. *Significant increase in promoter activity due to H2O2 treatment. # Significant reduction in promoter activity due to addition of SB 203580.

6. DISCUSSION

As discussed in the Introduction, a large body of evidence exists suggesting that Cav-1 is a tumor suppressor gene. Furthermore, it was shown that induction of premature senescence by Cav-1 could explain its tumor suppressor function since it has been shown that Cav-1 is up regulated by various stresses and it then plays a central role in the induction of SIPS. However, the molecular pathway involved in the up regulation of Cav-1 is not known. To elucidate this pathway, we carried out a 5' deletion analysis of the Cav-1 promoter region and EMSA which showed that two GC rich Sp1 consensus sites were important in up regulation of Cav-1 during SIPS. Further transfection studies showed that Sp-1 is binding to these sites.

Sp1 belongs to a large family of transcription factors, the Sp/KLF (Specificity Proteins/ Kruppler-like Factors) family. (171) Sp1 is the most ubiquitous and widely studied member of this family. Sp1 consensus sites have been found in the promoters of a number of cell growth and cell cycle related genes suggesting that Sp1 is important in regulation of these processes. Interestingly, Sp1 has been shown to have either positive or negative effects on cell growth and proliferation depending on the gene that it activates and the conditions under which this gene is being activated. For instance, $p21^{waf/cip1}$ promoter has Sp1 sites that are involved in inhibition of cell cycle and cell growth in many tissues. (152 – 154, 161)

Furthermore, as already discussed, it has been shown that caveolin is a tumor suppressor gene. Our lab has previously shown that could be through a) block in cellular proliferation and b) inhibition of cell cycle. (130) Thus, the present study showing that Sp1 up regulates caveolin-1 during oxidative stress is consistent with previous data.

The free radical/oxidative stress theory of aging proposes that:

a) Accumulation of free radicals with age and/or stress contributes to senescence at the cellular and organism level. (155, 156) We have already shown that caveolin-1 up regulation is necessary

and sufficient to cause senescence in tissue cultures in response to stress induced by sub cytotoxic levels of H_2O_2. (138) Interestingly, it has been shown that Cav-1 increases with aging. (157) In the present study, we show that this up regulation of caveolin in SIPS could be mediated by Sp1 through the identified consensus binding sites

b) Decreasing the levels of oxidative stress e.g. through increased scavenging of ROS by anti oxidants will retard aging. Endogenous anti oxidants such as glutathione along with exogenous dietary anti oxidants such as quercetin and vitamin C are believed to have a free radical scavenging action in vivo and are believed to delay this process. Decreased levels of these anti oxidants disturb the redox potential leading to oxidative stress and aging/ degenerative disease. (158 – 160) In the present study, we have shown that addition of quercetin, an antioxidant decreases the promoter activity of caveolin-1.These two findings thus add to the already large body of data available to support the free radical/ oxidative stress theory of aging.

Western blots and luciferase assay showed that there are increased levels of phosphorylated p38 during oxidative stress and this increase is essential for the up regulation of cav-1. This is in line with numerous studies that suggest that this pathway is activated during cellular stress. (162, 163) After activation, p38 MAPK has been shown to act on a variety of substrates. These include a number of protein kinases and transcription factors. (164) Through these downstream elements, p38 MAPK has been shown to have a number of effects. Significantly, recent studies have shown that these effects include cell cycle arrest (165-167) and cell senescence. (168 – 170) Furthermore, these investigators also suggested that decreased activity of p38 MAPK might play a role in loss of senescence as a tumor suppressor function resulting in the formation of tumors. Thus, the present study showing the role of p38 MAPK in up regulation of cav-1 is in line with existing evidence.

7. CONCLUSIONS

Numerous studies provided very strong evidence for the tumor suppressor capability of Cav-1. Moreover, it was also shown that Cav-1 normally negatively regulates the progress of the cell cycle. Our lab further showed that Cav-1 is up regulated during conditions of stress and this increase in Cav-1 levels was sufficient and necessary for the induction of premature senescence, possibly in cells with malignant potential also. We proposed that this might explain the tumor suppressor capability of Cav-1. To elucidate the molecular mechanisms leading to the up regulation of Cav-1 during stress, we carried out the present study using H_2O_2 to generate oxidative stress. Luciferase assays carried out with a series of luciferase reporter- Cav-1 promoter indicated that most of the activity during oxidative stress lies in the segments -91/-150 and -222/-372. Further analysis showed that both these segments had Sp1 consensus sites. EMSA and luciferase assays provided very strong evidence that Sp1 is in fact binding at these sites and that this binding is increased during oxidative stress. It can be concluded that the transcription factor binding to regulatory elements of Cav-1 gene in response to oxidative stress is Sp1. Interestingly, quercetin, a well known anti oxidant, reduced oxidant stimulated Cav-1 promoter activity at these sites showing that senescence induced by oxidative stress and the up regulation of cav-1 can be retarded by modulating the redox potential. Further more, when SB, a potent inhibitor of p38 MAPK was added, the up regulation of Cav-1 was abolished showing that this pathway plays a central role. In view of the data, we propose that the p38 MAPK pathway is stimulated in response to oxidative stress by yet unknown mechanisms. This pathway then increases the level and/or activity of the Sp1 transcription factor which leads to increased Cav-1 promoter activity during oxidative stress. This results in an increase in Cav-1 levels culminating in induction of stress induced premature senescence by the Caveolin-1 protein.

8. FUTURE STUDIES

The EMSA and the luciferase assays provide strong evidence that Sp1 is binding at the identified consensus sites. However, evidence showing the direct interaction between Sp1 and the identified consensus sequences is lacking. For this, confirmatory studies in the form of EMSA supershift assay, ChIP or DNA foot printing are required. These studies would provide conclusive evidence for the direct interaction between the Sp1 consensus sites and Sp1 transcription factor.

Previous studies have shown that the Sp/Kruppler family of transcription factors has a complex inter relationship. These studies showed that more than one member of the family may bind at the same site to regulate the expression of a gene, often with opposite effects. Sp3 has been shown to the Sp1 consensus sequence and down regulate a number of genes that Sp1 up regulates. (171-174) Also, Sp1 has been shown to form a complex with other transcription factors such as E2F and p53 during the regulation of cav-1 gene under other conditions. (150, 151) The role of other transcription factors, if any was not looked into in this study.

Lisanti et al who had first described the promoter sequence used in this study, suggested that there might be enhancer elements in the cav-1 gene up stream of the promoter, perhaps within the first exon or intron. (149) Since our study was limited to 1296 bp of the cav-1 promoter, we could not identify the enhancer sequences. Additional experiments would be needed to identify these possible enhancer sequences.

In the present study, we show the pathway leading to the up regulation of Cav-1. However, the exact mechanism by which Cav-1, after being up regulated leads to induction of senescence is not known. Future studies should look at these mechanisms.

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