Molecular mechanisms affecting expression of the NO receptor soluble Guanylyl Cyclase (sGC)

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TABLE OF CONTENTS

I Introduction

1 Historical Overview

After the discovery of 3',5'-cyclic adenosine monophosphate (cAMP) as the first second messenger molecule that regulates many important cell functions (Sutherland EW, 1958) guanosine 3',5'-monophosphate (cGMP) became the second identified member of this important class of signaling molecules, the cyclic nucleotides (Ashman DF, 1963). cGMP is involved in the regulation of vision, vasodilatation, platelet aggregation, smooth muscle cell proliferation, cellular ion homeostasis, synaptic plasticity and other important physiological processes (Carvajal JA, 2000);(McDonald LJ, 1995);(Sausbier M, 2000).

Cyclic GMP is synthesised by the guanylyl cyclase family of enzymes. It was established by the mid-1970s that guanylyl cyclase activity is present in both the soluble and particulate fractions of most cells (Schultz G, 1969);(White AA, 1969);(Hardman JG, 1969);(Ishikawa E, 1969), and that these activities are due to different proteins (Garbers DL, 1974);(Kimura H, 1974);(Kimura H, 1975a). Therefore, guanylyl cyclases are divided into two subfamilies: particulate and soluble guanylyl cyclases (sGC) (Chrisman TD, 1975);(Garbers DL., 1990).

1.1 Role of Nitric Oxide (NO)

While a number of peptide hormones that stimulate particulate guanylyl cyclase were known for some time (Waldman SA, 1985);(Murad F, 1987), the nature of the physiological regulator of sGC remained for long time enigmatic. Already in the 1970s, NO-releasing compounds were found to be potent activators of NO-sensitive GC (Arnold WP, 1977);(Bohme E, 1978). Similarly, nitrovasodilators like glyceroltrinitrate or isosorbid dinitrate therapeutically used for the treatment of coronary heart disease act by stimulation of the enzyme. These nitrates do not

release NO spontaneously; instead, they have to undergo bioactivation that either yields NO or nitrosothiols.

Despite the stimulatory effect of these NO-containing compounds, the physiological significance of NO-induced activation of the enzyme did not become apparent until the identification of endothelium-derived relaxing factor (EDRF) as NO (Ignarro LJ, 1987);(Palmer RM, 1987). Formation of EDRF had been shown to occur in endothelial cells in response to vasodilatory agonists such as acetylcholine, histamine, or bradykinin, leading to vasodilation via the activation of NO-sensitive GC in smooth muscle cells (Forstermann U, 1986). After the discovery of NO in the vascular system, NO formation was reported to occur throughout the body (Moncada S, 1995). The enzymes responsible for the synthesis of NO were identified and termed NO synthases of which three isoforms are known to date (neuronal NOS-NOS I, inducible NOS-NOS II, endothelial NOS-NOS III). These enzymes catalyze the oxidative deamination of the amino acid L-arginine, wherein by the consumption of 1.5 mol of NADPH and 2 mol of oxygen, a mol of NO is formed (Griffith OW, 1995). From two sequential monooxygenase reactions the final products NO and L-citrulline are synthesized (Marletta MA, 1988). The endothelial NO synthase is a constitutively expressed enzyme that shows a low basal activity, which is increased however by hormonal induced rise in the cytosolic Ca^{2+}-concentration which is regulated by calmodulin (Busse R, 1990);(Lamas S, 1992). The endothelial NO synthase is also activated by Akt/PKB-dependent phosphorylation triggered by shear forces exerted on the endothelial cells (Dimmeler S, 1999).

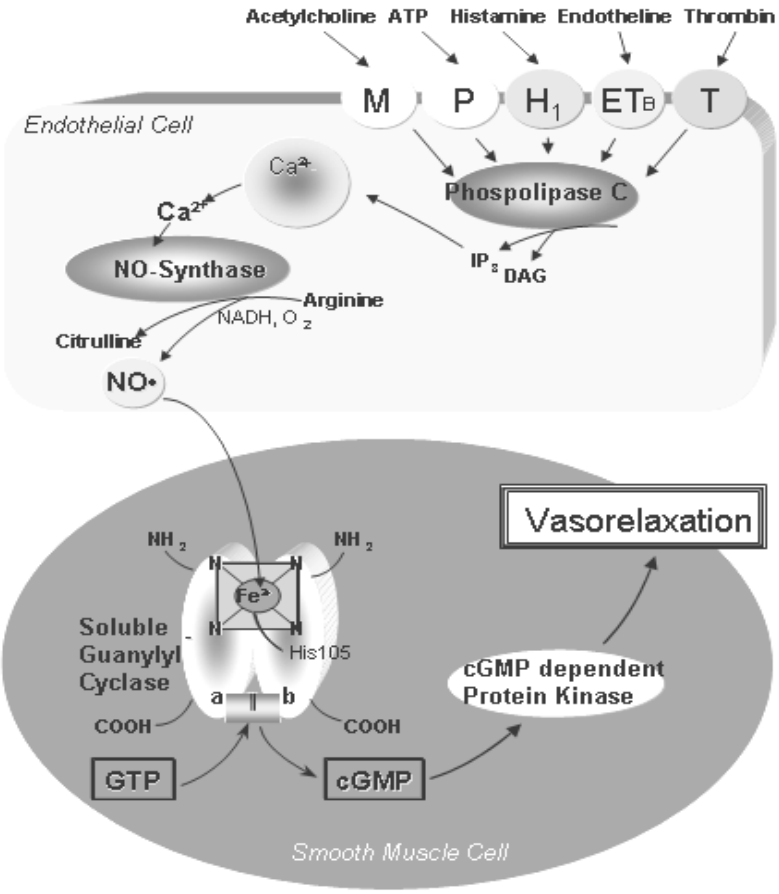


Figure 1. **Current scheme for endothelium-dependent relaxation**. Endothelial cells possess receptors on their cell membrane, for example for acetylcholine (M), ATP (P), histamine (H1), endothelin (ETB) and thrombin (T). Through G-proteins these factors can induce the activation of phospholipase C, which catalyzes the conversion of phosphatidylinositol-4, 5-bisphosphate (PIP_2) in inositol-1, 4, 5-triphosphat e (IP_3) and diacylglycerine (DAG). IP_3 sets Ca^{2+} free from intracellular stores. This leads to the activation of the Ca^{2+}/calmodulin-dependent endothelial NO synthase (NOS III), which forms NO and L-citrulline from oxygen and L-arginine. NO diffuses into the smooth muscle cells and stimulates the soluble guanylyl cyclase (sGC), which leads to cGMP accumulation and activation of the cGMP dependent protein kinases (G-kinases) which leads to vasorelaxation.

In-vivo investigations indicate that endothelial NO plays an important role in vascular homeostasis in humans and mammals (Furchgott RF, 1989);(Ignarro LJ, 1987);(Moncada S, 1991);(Rees DD, 1989);(Vallance P, 1989). Consequently disturbances of the NO /cGMP system lead to

diseases with vascular dysfunction. Another important mechanism is the reaction of NO with the superoxide radical leading to the formation of peroxynitrite (Beckmann JS, 1994) which in turn can lead to the nitration of other compounds such as prostacycline synthase (Zou MH, 1996). NO-mediated signaling has been implicated in vasodilatation, inhibition of leukocyte adhesion and platelet aggregation, regulation of endothelial permeability, neuronal signaling in the peripheral and central nervous system, and other processes (Zhuo M, 1995);(Walter U., 1989);(Rapoport RM, 1983);(Moncada S, 1995).

1.2 Role of cGMP

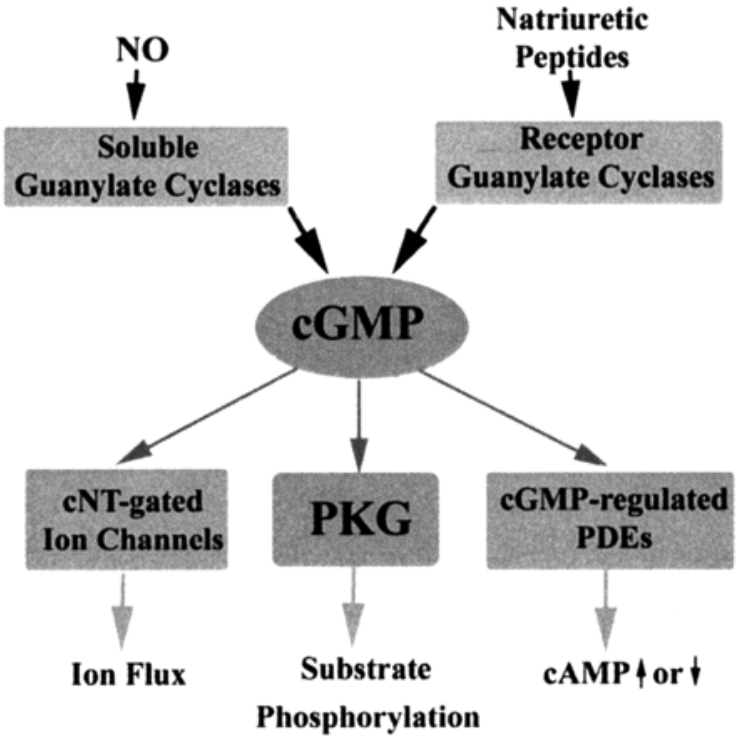


Figure 2. Cyclic GMP signaling pathway. Cyclic GMP is synthesized by soluble guanylate cyclases in response to NO or by receptor guanylate cyclases, which are activated by natriuretic peptides, for example. Depending on the cell type, cGMP has several intracellular targets in addition to cGMP-dependent protein kinases (PKG).

cGMP mediates its effects through four cellular proteins: cGMP dependent protein kinases (PKG), cyclic nucleotide-gated cation channels (CNG), cAMP dependent protein kinases (PKA) and phosphodiesterases (PDE) (Lucas KA, 2000).

The PKGs are serine/threonine kinases, which are activated by cGMP. Two types of PKG are well known: PKG I and PKG II. The PKG I is a cytosolic 76-kDa homodimer. It is expressed in most tissues and more strongly particularly in the cerebellum, in the blood cells and in the smooth muscle cells. Two isoforms of the PKG I were described, PKG PKG Iα and PKG Iβ. Most tissue with the exception of uterus, express both isoforms (Lohmann SM, 1997);(Tamura N, 1996). In mice, in which the gene for PKGI was switched off, vascular, intestinal, and erectile dysfunctions were described (Hedlund P, 2000);(Pfeifer A, 1998).

The PKG II is a 86 kDa protein, which is also expressed in a large number of tissues though not in the cardiovascular system (Jarchau T, 1994);(Lohmann SM, 1997);(Uhler MD., 1993). The only well-known target substrate of the PKG II is the cystic fibrosis transmembrane conductance regulator (CFTR) (Vaandrager AB, 1997). In knockout studies in mice intestinal secretion defects and dwarf stature were observed.

CNG channels are a family of voltage-gated cation channels expressed in a variety of cells. There are five isoforms of the CNG (Biel M, 1999);(Frings S., 1997);(McCoy DE, 1995);(Misaka T, 1997). The isoforms CNG 1 and CNG 3 are involved in cGMP-mediated phototransduction at the level of rod and cone photoreceptors that regulates neurotransmission within the retina.

Although cGMP is not the classical substrate for PKA, it can still activatePKA. The regions responsible for binding to the cyclic nucleotides in PKA are very similar to PKG. However the selectivity for the activation of PKA by cGMP is less by a factor of 50 as compared to PKG (Lohmann SM, 1997);(Pfeifer A, 1998).

The phosphodiesterase (PDE) is the last group of the cGMP activated signal molecules. These enzymes catalyze the conversion from cAMP or cGMP to 5'-AMP or 5'-GMP, which do not possess any biological activity. Altogether 13 PDE isoenzymes have been described to date, of which the first five are: the calcium/calmodulin dependent PDE I, the cGMP stimulated PDE II, the one inhibited by cGMP (PDE III), cAMP specific PDE IV and the cGMP specific PDE V (Pfeifer A, 1998);(Schmidt HH, 1993). Of particular importance is the cGMP specific PDE V, which is central for the signal transduction in the retinal rods and cones.

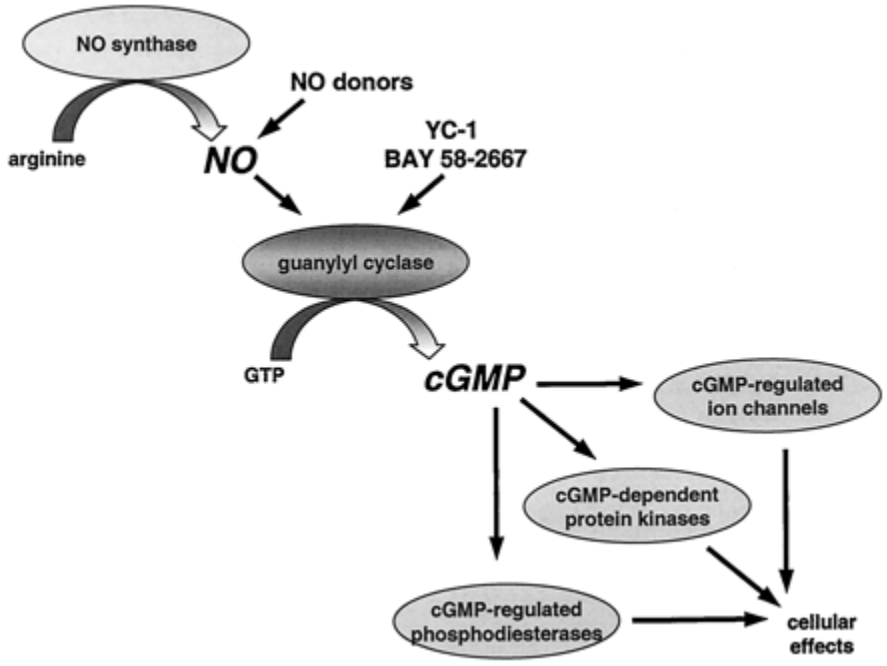


Figure 3. NO/cGMP signaling cascade. NO endogenously produced by NO synthases or released from exogenously applied NO donors activates NO-sensitive sGC and leads to increased synthesis of cGMP. This intracellular messenger in turn modulates the activity of cGMP-dependent kinases, cGMP-gated ion channels, and cGMP-regulated phosphodiesterases. These effectors are involved in the regulation of several physiological functions in the cardiovascular and nervous systems. YC-1 and BAY 58-2667 represent new classes of activators of NO-sensitive GC (Friebe A, 2003).

2 Guanylyl Cyclase

2.1 Particulate Guanylyl Cyclase (pGC)

Seven eutherian mammalian pGC isoforms (GC-A to GC-G) have been identified (Table 1). They exhibit highly conserved domain structures, including (1) an extracellular binding domain at the N terminus that in some cases binds defined ligands (GC-A, -B, -C), (2) a single transmembrane domain, (3) a cytoplasmic juxtamembrane domain, (4) a regulatory domain that shares significant homology with protein kinases, (5) a hinge region, and (6) a C-terminal catalytic domain (Figure 4). Isoforms expressed in intestinal mucosal cells (GC-C) and in sensory organs (GC-D, -E, -F) also possess a C-terminal tail. Based on their ligand specificities, pGCs have been classified as (1) natriuretic peptide receptors, (2) intestinal peptide-binding receptors, and (3) orphan receptors (Table 1). pGCs are expressed in almost all tissues in placental mammals (Table 1).

Table 1: Particulate guanylyl cyclase isoforms, ligand and co-factor specificities, chromosomal localisation and tissue distribution.

	Ligand(s)	Cofactors	Chromosome Location	Tissue Distribution
GC-A (NPR-A)	ANP, BNP	ATP	1p21-p22	Adipose tissue, adrenal gland, ileum, kidney, placenta; adrenal gland, cerebellum, heart, kidney, pituitary; lamina propria; cochlea; thymus ovary
GC-B (NPR-B)	CNP	ATP	9p12-p21	Placenta; adrenal medulla, cerebellum, pituitary; adrenal gland, aorta, atrium, cerebellum, lung, intestine, pituitary, testis, ventricle; uterus/oviduct; thymus; ovary
GC-C	ST, Guanylin, Uroguanylin		12p12	Intestinal mucosa; regenerating liver
GC-D	?		11p15.4 or 11q13-q14.1	Olfactory epithelium
GC-E (Ret GC-1)	?	GCAP1,2,3	17p13.1	Retina, pineal gland
GC-F (Ret GC-2)	?	GCAP 2,3	Xq22	Retina
GC-G	?		10q24-q26c	Intestine, kidney, lung, skeletal muscle

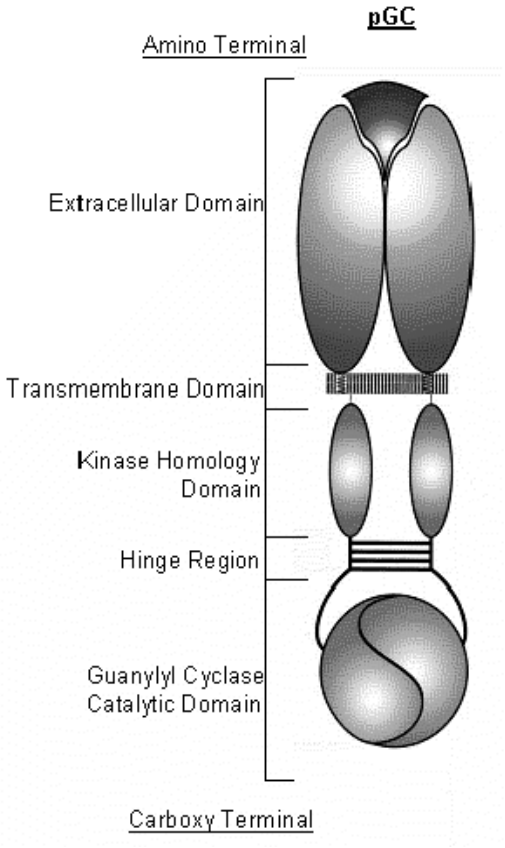


Figure 4: Domain structure of particulate guanylyl cyclases (pGC). The pGCs are homodimeric proteins that possess extracellular ligand binding domain, a transmembrane, kinase homology domain, a hinge region and a GC catalytic domain. The pGC illustrated is a homodimer modeled after GC-A and B and possess a single ligand binding site formed by two extracellular amino terminal domains. GC-C, D, E and F possess a carboxy terminal tail that is not depicted here (Lucas et al., 2000).

2.2 Soluble Guanylyl Cyclase (sGC)

The purification of guanylyl cyclase from the 100,000xg centrifugation supernatant of tissue extracts showed that the soluble isoform is a heterodimer, consisting of an α and a β subunit exhibiting a molecular weight of ~70 kDa and ~73 to 82 kDa respectively (Kamisaki Y, 1986). It was another four years, until sGC in pure form was isolated from bovine or rat lung, the primary sequence was obtained and the catalytic domain was identified (Nakane M, 1988);(Koesling D, 1988);(Nakane M, 1990).

The soluble guanylyl cyclase (sGC) is found in the cytoplasm of nearly all mammalian cells (not in red blood cells) and mediates a large range of important physiological processes like the inhibition of platelet aggregation (Buechler WA, 1994), relaxation of smooth muscle (Warner TD, 1994), vasodilation, neuronal signal transduction and immune modulation (Collier J, 1999).

2.2.1 Isotypes of sGC

Table 2: Soluble guanylyl cyclase isoform chromosome localisation and tissue distribution

Subunit	Chromosome Location	Tissue Distribution
α1 (α3)	4q31.3-q33	Lung; cerebellum, cerebrum, heart, kidney, liver, lung skeletal muscle; kidney
α2	11q21-q22	Brain, retina; kidney; placenta
β1 (β3)	4q31.3-q33	Lung; cerebellum, cerebrum, heart, kidney, liver, lung skeletal muscle
β2	13q14.3	Kidney, liver; kidney

Presently, five different subunits of sGC are well known to exist: α-1, α-2, α-i, β-1 and β-2. The different subunits are expressed in a tissue and development-specific manner (Hobbs AJ., 1997);(Giuili G, 1992). The chromosomal loci of the genes encoding isoforms of guanylyl cyclase and their ligands have been mapped in the human and/or the mouse (Table 2) and are unlinked and scattered

throughout the genome, with notable exceptions. For the rat so far only the subunits α-1, α-2, β-1 and β-2 have been described. The amino acid similarity between humans and rat for the α-1 subunit is about 84% (chromosomal location: 2q31-q33), for the α-2 subunit, 90%, for the β-1 subunit, 99% (chromosomal location: 2q31-q33) and for the β-2 subunit 81% (chromosomal location: 5q36) (Denninger JW, 1999).

The most abundant subunits are α-1 and β-1, which are found in many tissues. Concomitant expression of both subunits is required for catalytic activity (Kamisaki Y, 1986);(Harteneck C, 1990);(Buechler WA, 1991);(Wedel B, 1995). These subunits were first cloned from rat and bovine lung (Koesling D, 1988);(Koesling D, 1990);(Nakane M, 1990);(Nakane M, 1988). Depending upon the species, the molecular weight of the α-1 subunit ranges from 70-82 kDa and that of the β-1 subunit between 70-73 kDa. The rarer α-2 subunit (82 kDa) has been found to be expressed only in the human foetal brain (Harteneck C, 1991) and placenta (Russwurm M, 1998). sGC β-2 has never been found at the protein level; its mRNA was detected in rat kidney and in liver (Yuen PS, 1990).

Although the β-2 subunit can form a heterodimer with α-1, this holoenzyme exhibits lower specific activity compared with α-1/β-1. Thus, NO-stimulation of COS-7 cells cotransfected with α-1/β-1 resulted in three times more cGMP production than in cells transfected with α-1/β-2 (Gupta G, 1997). Coexpression of β-2 with α-1/β-1 decreased the formation of the α-1/β-1 heterodimer, presumably due to competition between β-1 and β-2 for binding to α-1. These data support the hypothesis that expression of β-2 may serve to regulate α-1/β-1 sGC activity. In fact, expression of β-2 has been suggested to play a role in the pathogenesis of hypertension in the Dahl rat (Gupta G, 1997). The ~82-kDa α-2 cloned from human fetal brain forms heterodimers with β-1 or β-2, but has lower affinity for β-1. Indeed, α-2/β-1 has a lower specific activity than α-1/β-1 (Harteneck C, 1991). Two other subunits of human sGC, α-3 (82 kDa) and β-3

(70 kDa), have been cloned from adult brain; however, later re-examination of sequence data revealed these isoforms as artifacts. Homodimerization of sGC α-1 or sGC β-1 subunits has also been demonstrated, although the homodimers were inactive (Zabel U, 1999). An intriguing exception to this rule seems to be β-2, which has been found to be active as a homodimer as determined in expression experiments in Sf9 cells (Koglin M, 2001). Recently AGAP1 (the prototype of an ArfGAP protein with a GTPase-like domain, Ankyrin repeats, and a pleckstrin homology domain) has been identified as a novel and specific interaction partner of sGC *in vitro* and *in vivo* (Meurer S, 2004). It has been demonstrated that AGAP1 associates with both the α-1 and β-1 subunits of sGC and that complex formation between these proteins is modulated by tyrosine phosphorylation. Association with AGAP1 does not affect the enzymatic capacity of sGC nor does it alter its NO sensitivity. It is possible that AGAP1, by binding to sGC, may help regulate the intracellular distribution of sGC and thus the local delivery of cGMP in mammalian cells.

RNA splicing also contributes to the heterogeneity of sGC subunits. A variant of the α-2-subunit, α-2_i, was identified in a number of cell lines and tissues by PCR using primers based on conserved sequences in the catalytic domain of mammalian guanylyl cyclase (Behrends S, 1995). The subunit α-2_i is produced through alternative splicing of RNA that adds 31 amino acids to the catalytic domain, with homology to a region within the catalytic domain of adenylyl cyclases. Previous studies demonstrated sGC catalyzed the conversion of ATP to cAMP (Mittal CK, 1977a). The region of homology in α-2_i was postulated to increase the ability of this isoform of sGC to utilize ATP as a substrate and produce cAMP (Behrends S, 1995). However, coexpression of the α-2_i/β-1 in Sf9 cells abolished the ability of sGC to produce cAMP (Behrends S, 1995). In addition, Sf9 cells transfected with α-2_i/β-1 are devoid of guanylyl cyclase activity, whereas coexpression of α-2/β-1 results in production of cGMP in these cells, suggesting the α-2_i-subunit can compete with α-2 for binding to β-1 and act as a

dominant negative inhibitor. Expression of this subunit may serve as a mechanism to regulate sGC activity in specific cells (Behrends S, 1995).

2.2.2 Location of sGC genes

The genes encoding human sGC subunits α-3 (equivalent to α-1) and β-3 (equivalent to β-1) have been mapped to chromosome 4q32 (Giuili G, 1993). Because both subunits are required in a 1:1 stoichiometry for activity, their common chromosomal locus may imply a coordinated regulation of gene expression. The genes encoding α-1 and β-1 sGC subunits in the medaka fish are organized in tandem within a 34-kb span (Mikami T, 1999). The activity of the 5'-upstream region of each of the medaka fish genes was analyzed using green fluorescent protein reporter constructs expressed in medaka embryos (Mikami T, 1999). Although the α-1 upstream region promoted expression of green fluorescent protein, the β-1 5' region was insufficient, suggesting expression of the α-1 and β-1 genes is coordinated. However, the α-2 subunit, which also can form an active dimer in vitro with β-1, is encoded by a gene on chromosome 11 (Yu F, 1996). That α-2 and β-1 subunits dimerize under physiological conditions argues against the requirement for coordinated regulation of expression of α-1 and β-1 subunits (Russwurm M, 1998). The genomic organisation of these subunits has recently been characterised in mouse and was found to be similar to that in the medaka fish (Sharina IG, 2000). The α-1 sGC gene is approximately 26.4 kb and contains nine exons, whereas the β-1 sGC gene spans 22 kb and consists of 14 exons. Both mouse genes are localized on the third chromosome, band 3E3-F1, and are separated by a fragment that is 2% of the chromosomal length. Genomic DNA corresponding to the β subunit (GCS β) gene from *Anopheles gambiae* (Caccone A, 1999), and for both the subunits from Drosophila (Shah S, 1995) have also been cloned and sequenced and the gene structure has been described.

2.2.3 Structure of sGC

A comparison of the primary structure shows that the subunits can be divided into three domains: a C-terminal catalytic domain, a central part, and an N-terminal region. The catalytic C-terminal domains of each subunit of NO-sensitive GC display the highest degree of homology (Wedel B, 1995). These domains are also very similar to the respective regions in the peptide-activated, membrane-bound guanylyl cyclases and in the adenylyl cyclases (AC). The catalytic domains of AC and NO-sensitive GC are structurally closely related as shown by the functional expression of AC/GC heterodimers (Weitmann S, 1999) and the conversion of substrate specificity (ATP vs. GTP) by the mutation of only three amino acids (Sunahara RK, 1998).

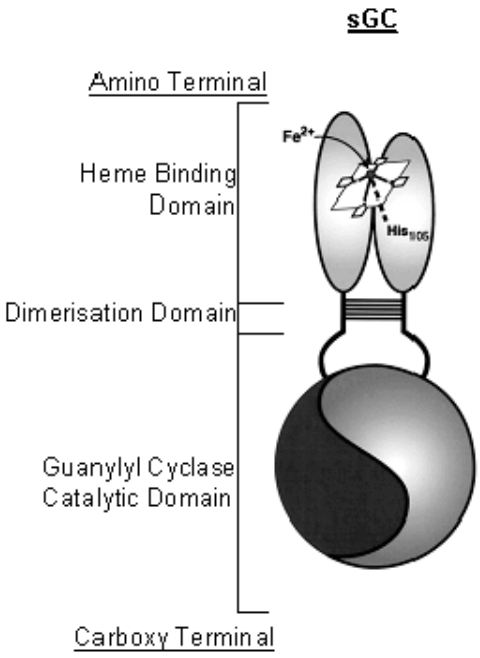


Figure 5: Domain structure of soluble guanylyl cyclase (sGC). sGCs are heterodimers possessing amino terminal regulatory domains containing a heme prosthetic group with a ferrous (Fe^{2+}) core that forms an imidazole axial bond with His-105 of the β-subunit. In addition, sGCs possess homodimeric domains and carboxyl terminal catalytic domains that form one active and one inactive catalytic site (Lucas et al., 2000).

The central regions preceding the catalytic domains show considerable homology to the membrane-bound enzymes (Wilson EM, 1995). In analogy, these regions are probably involved in the dimerization of the subunits, although detailed investigation is still missing. Recently there has been evidence that the dimerization region of β-1 extends over 205 residues of its regulatory and central domains and that two discontinuous sites of 41 and 30 residues, respectively, facilitate binding of β-1 to the α-1 subunit of sGC (Zhou Z, 2004).

The N-terminal regions of the subunits of NO-sensitive sGC comprise the heme-binding domain. NO-sensitive sGC is a hemoprotein and presence of the prosthetic heme group is mandatory for the activation of the enzyme by NO (Craven PA, 1978);(Ignarro LJ., 1990). Removal of the heme group abolishes NO-induced activation, which can be restored after reconstitution of NO-insensitive sGC with heme (Ignarro LJ, 1986);(Foerster J, 1996). The heme stoichiometry has been agreed to be one mole per mole sGC heterodimer. Both, the α and β subunits are required for proper binding and orientation of the heme group. Sequential truncation showed that the subunits contribute unequally to heme binding with the α-1 subunit playing a more important role (Koglin M, 2003); in addition, homodimers of the N-terminal part of the β-1 subunit (the first 385 amino acid) expressed in bacteria were shown to bind heme in a manner similar to the wild-type enzyme (Zhao Y, 1997). A histidine residue (His-105) within this region of the β-1 subunit was shown to bind to the heme iron, thereby acting as the proximal ligand. Deletion of His-105 has been shown to abrogate NO activation of the enzyme (Wedel B, 1994). Mutation of this histidine led to the generation of an NO-insensitive, heme-depleted enzyme with intact basal activity. In addition, two conserved cysteines adjacent to the His-105 on the β-1 subunit appear to play a role in the formation of the proper heme pocket. Mutation of these residues led to loss of enzyme-bound heme and NO responsiveness, which could be regained after heme reconstitution (Friebe A, 1997).

2.2.4 Activators of sGC

Already in the 1970s, NO-releasing compounds were found to be potent activators of sGC (Arnold WP, 1977);(Bohme E, 1978). Similarly, nitrovasodilators like glyceroltrinitrate or isosorbid dinitrate therapeutically used for the treatment of angina pectoris and coronary heart disease act by stimulation of the enzyme. These nitrates do not release NO spontaneously; instead, they have to undergo bioactivation that either yields NO, nitrosothiols, or another sGC activating NOx species.

Despite the stimulatory effect of these NO-containing compounds, the physiological significance of NO-induced activation of the enzyme did not become apparent until the identification of endothelium-derived relaxing factor (EDRF) as NO (Palmer RM, 1987);(Ignarro LJ, 1987). Formation of EDRF had been shown to occur in endothelial cells in response to vasodilatory agonists such as acetylcholine, histamine, or bradykinin, leading to vasodilation via the activation of NO-sensitive sGC in smooth muscle cells (Forstermann U, 1986). After the discovery of NO in the vascular system, NO formation was reported to occur throughout the body (Moncada S, 1995). The enzymes responsible for the synthesis of NO were identified and termed NO synthases of which three major isoforms are known to date. The inducible isoform produces relatively high NO concentrations and thereby exhibits direct toxic effects. The enzyme is mainly expressed in macrophages and plays a role in the nonspecific immune response. The neuronal and endothelial NO synthases are constitutively expressed enzymes that are regulated by the intracellular calcium concentration. The endothelial NO synthase is also activated by Akt/PKB-dependent phosphorylation triggered by shear forces exerted on the endothelial cells (Dimmeler S, 1999). Both isoforms produce relatively low NO concentrations (1 to 100 nmol/L). At these low concentrations, NO functions as a signaling molecule, and most of its effects are mediated via the activation of NO-sensitive sGC.

Activation occurs via the binding of NO to the prosthetic heme group of sGC. It is assumed that in the NO free state, iron is pentavalent and bound to histidine-105 of sGC α-1. After NO binding, the iron-histidine bond breaks, which produces conformational changes in the sGC molecule and subsequently enzyme activation (Koesling D., 1999). Indeed, by recording of spectral profiles of sGC before and after NO addition, the changes in iron coordination and the break of the iron-histidine bond were confirmed (Schelvis JP, 1998);(Sharma VS, 1999). The essential role of this bond break was confirmed by the observation that another heme-binding molecule, CO, is a weak sGC activator. Unlike NO, CO does not break the iron-histidine-105 bond but evokes hexavalent ligation of iron (Stone JR, 1994);(Burstyn JN, 1995). It is assumed that the failure of CO to break the iron-histidine bond is the reason for its weak sGC-activating potency. However, combined action of CO and another NO-independent sGC activator, YC-1 (see below), results in activation of sGC comparable to that of NO without loss of 6-coordinated state of heme (Stone JR, 1998);(Denninger JW, 2000).

Additional evidence for the role of heme interaction with the protein for sGC activation is the ability of protoporphyrin IX (PPIX), an iron-free heme, to stimulate hemedeficient sGC (Ohlstein EH, 1982). NO or CO do not stimulate heme-deficient sGC. Interestingly, YC-1 could potentiate the PPIX-stimulated sGC activity, but no shift of the concentration-response curve as seen with NO or CO was observed (Friebe A, 1996);(Friebe A, 1998a).

Besides the well-established NO/heme-mediated stimulation, two novel mechanisms of activation have been identified for NO-sensitive sGC, indicating considerable therapeutic potential. First, the activator YC-1 (Figure 1 and 4) was shown to act as an "NO sensitizer," greatly enhancing the sensitivity of GC toward NO (Friebe A, 1996);(Friebe A, 1998a). Derivatives of YC-1 and other novel substances have been identified (Lee FY, 2001);(Straub A, 2001);(Stasch JP, 2002a);(Miller LN, 2003). By increasing the NO responsiveness under pathological conditions characterized by reduced NO release, these NO

sensitizers may become important drugs in the treatment of various cardiovascular diseases. The effects of YC-1 on NO/cGMP signaling have been investigated in a variety of cells and tissues. In vascular smooth muscle cells, YC-1 was reported to increase cGMP levels and to induce a concentration-dependent relaxation of endothelium-denuded rat aortic rings (Mulsch A, 1997);(Wegener JW, 1997);(Galle J, 1999). Other tissues in which YC-1 induced cGMP elevations include guinea pig trachea (Hwang TL, 1999), guinea pig colon (Hallen K, 2001), corpus cavernosum (Brioni JD, 2002), and pig urethra (Schroder A, 2002). In endothelial cells, the YC-1 induced cGMP increases were most pronounced as in these cells YC-1 did not only stimulate NO-sensitive GC directly but also potentiated the stimulatory effect of the endogenously synthesized NO (Wohlfart P, 1999). In platelets, YC-1 was shown to inhibit adhesion and aggregation (Wu CC, 1995);(Teng CM, 1997);(Friebe A, 1998b). In these cells, YC-1 led to a drastic, over 1000-fold increase in cGMP in the presence of NO. Closer analysis revealed that YC-1 applied in higher concentrations (10-100 µM) inhibited various PDEs (Friebe A, 1998b);(Galle J, 1999). Due to the high catalytic rate of PDEs, some of the YC-1 effects observed in intact cells may be additionally caused by the inhibition of PDEs rather than solely by the stimulation of NO-sensitive GC.

A second novel mechanism of activation has been shown to occur using the substance BAY 58-2667 (fig. 1 and 4) (Stasch JP, 2002b). This compound, an amino dicarboxylic acid, alone led to a moderate activation of sGC; this stimulation was additive to that of NO, which is in contrast to the potentiation seen with YC-1. Surprisingly, BAY 58-2667 stimulated the heme-oxidized or heme-depleted purified enzyme almost 200-fold. This indicates that BAY 58-2667 may be able to substitute for the heme group or stabilize the heme-free conformation; the occurrence of the heme-free form of NO sensitive GC under physiological conditions remains to be elucidated. Photoaffinity labeling and binding studies suggest binding of the compound to both subunits of NO-sensitive GC.

A great number of other substances have been considered as possible physiological sGC regulators. However, after discovery of the role of NO in sGC activation, many (if not all) of these effects could be explained via effects on NO bioavailability or stability.

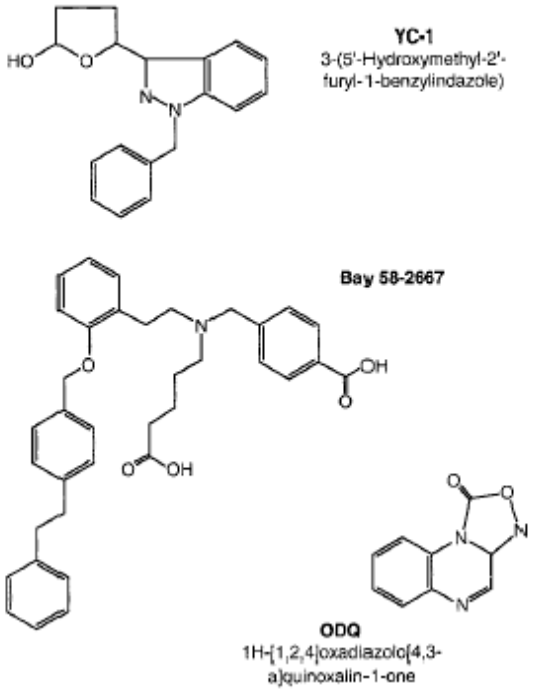


Figure 6: Pharmacological compounds acting on NO-sensitive-GC. YC-1 and BAY 58-2667 are novel activators of NO-sensitive-GC that increase cGMP production independent of NO. YC-1 potentiates the stimulatory action of NO and thus serves as “NO sensitizer”: BAY 58-2667 preferentially activates the heme-depleted form of the enzyme. ODQ, in contrast, inhibits NO-sensitive-GC by oxidizing the heme iron leaving basal enzyme activity intact (Friebe A, 2003).

2.2.5 Inhibitors of sGC

Several substances have been found to inhibit NO-sensitive GC. The quinoxalin derivative 1H-[1,2,4] oxadiazolo [4,3-a]-quinoxalin-1-one (ODQ; Figure 6) was shown to be a potent and selective inhibitor of NO-sensitive GC in brain slices (Garthwaite J, 1995). ODQ does not lead to the inhibition of the membrane-bound guanylyl or adenylyl cyclases and interferes with the stimulation of NO synthase and other heme proteins only at about 100-fold higher concentrations than required for inhibition of sGC (Feelisch M, 1999). Therefore, ODQ represents an important tool to discriminate cGMP-dependent and cGMP-independent effects of NO. Inhibition of NO-sensitive GC by ODQ has been demonstrated in a variety of other cells and tissues (Abi-Gerges N, 1997);(Brunner F, 1996);(Garthwaite J, 1995);(Moro MA, 1996). Studies with purified NO-sensitive GC revealed that ODQ binds in an NO-competitive manner and leads to an apparently irreversible inhibition of the stimulated enzyme leaving basal activity unchanged. Spectral analysis suggests oxidation of the heme iron as underlying mechanism of the inhibitory effect (Schrammel A, 1996);(Zhao Y, 2000). The notion that the heme iron is the target of ODQ is confirmed by the finding that ODQ is not able to inhibit the stimulation of NO-insensitive GC induced by the iron-free protoporphyrin IX (Koesling D, 1999). A derivative of ODQ, NS 2028 (oxadiazolo(3,4-d)benz(b)(1,4)oxazin-1-one), has been published to have properties similar to those of ODQ (Olesen SP, 1998).

Other inhibitors such as methylene blue and LY-83583 show less specificity than ODQ and, eg, inhibit the olfactory cyclic nucleotide-gated ion channel (Leinders-Zufall T, 1995) or interfere with NO formation and NO release from NO synthases (Mayer B, 1993); therefore, ODQ should be preferred as specific sGC inhibitor.

2.2.6 Role of divalent cations

sGC requires divalent metal cations for catalytic activity. Both Mg^{2+} and Mn^{2+} can maintain catalytic activity of sGC probably via binding to the catalytic site or forming a complex with nucleotide substrate (Me-GTP) (Frey WH 2nd, 1977);(Mittal CK, 1977b). Interestingly, these two metals differ in their effects on basal and stimulated sGC activity. While Mg^{2+} is required for maximal stimulation of sGC with NO, basal activity of the enzyme is higher in the presence of Mn^{2+} (Craven PA, 1976);(Kimura H, 1976). Exact mechanisms of these different actions of both cations are unclear.

Another interesting point in the regulation of sGC by divalent cations is the role of calcium. It has been demonstrated that Ca^{2+} inhibits recombinant sGC (Parkinson SJ, 1999) and sGC purified from bovine lung (Kazerounian S, 2002). Inhibition of sGC by Ca^{2+} in rat anterior pituitary cells has been also demonstrated (Andric SA, 2001). The mechanism of the calcium effect on sGC activity is not completely elucidated. Recently, sGC has been found to specifically bind Ca^{2+}, and the existence of two different binding sites for divalent cations on the enzyme has been postulated (Kazerounian S, 2002). Ca^{2+} is able to inhibit purified sGC via binding to a high affinity-binding site in the concentration range that is in the physiological range of intracellular Ca^{2+} concentration in activated cells (100 - 500 nM). At a Ca^{2+} concentration of about 10 µM the inhibition of sGC was complete, which was explained by the binding of Ca^{2+} to a low affinity site (Kazerounian S, 2002). It has been also demonstrated that Mg^{2+} was not able to prevent the binding of Ca^{2+} to a high affinity-binding site and thus also partly reversed Ca^{2+}-dependent sGC inhibition via displacement of Ca^{2+} from a low affinity-binding site. Interestingly, Mn^{2+} was able to prevent the inhibitory effect of Ca^{2+} on sGC (Kazerounian S, 2002). Demonstration of Ca^{2+}-dependent inhibition of sGC is an intriguing observation, since cGMP serves in many cases as a negative regulator of the Ca^{2+} concentration and the effects of these substances (Ca^{2+} and cGMP) are very often antagonistic.

3 Regulation of expression of sGC

There are many studies over the last several years showing that sGC expression can be finely regulated at the transcriptional and post-transcriptional level in different cell types.

There is increasing evidence of a developmental regulation of sGC, with the α-1 subunit being expressed prenatally in the rat brain (Smigrodzki R, 1996) and changes in the expression pattern observed in rat heart during post-natal development (Behrends S, 2002). Developmental changes of sGC expression have also been observed in rat ovary, pulmonary arteries and lungs. A regulated, cell- specific pattern of sGC expression in the rat ovary has been observed and is consistent with roles for cGMP in modulating ovarian functions (Shi F, 2004). Inhaled NO is known to influence the contractile state of pulmonary arteries most likely by activation of sGC in smooth muscle cells and an increase in sGC immunoreactivity in endothelial cells and a reciprocal decrease in smooth muscle cells was observed with postnatal development (Behrends S, 2001). Pulmonary sGC is increased during the perinatal period, which suggests an important role for NO-cGMP signal transduction in the perinatal regulation of pulmonary epithelial function and bronchial tone (Bloch KD, 1997).

The availability of NO plays an important role in regulation of sGC expression. It was demonstrated that NO donors reduce sGC levels in kidney medullary interstitial (Ujiie K, 1994) and smooth muscle cells (Filippov G, 1997). It was also demonstrated that agents inducing the expression of the constitutively active high output form of NOS, NOS-II, produce downregulation of sGC mRNA, protein, and activity levels (Papapetropoulos A, 1996a);(Takata M, 2001).

The mechanism of sGC regulation by NO and participation of cGMP in this process seems to be not completely resolved. Filippov and co-worker have investigated the mechanism of this down-regulation of sGC. They demonstrated

that reduction of sGC mRNA is mediated by a decrease in its stability, an effect that is cGMP dependent in smooth muscle cells (Filippov G, 1997). cGMP analogues or cGMP-elevating agents down-regulate sGC mRNA levels also in other cell types (Ujiie K, 1994);(Papapetropoulos et al., 1996);(Filippov G, 1997). However, cGMP-independent regulation of sGC protein levels in porcine endothelial and smooth muscle cells has also been observed (Ibarra et al., 2001).

Expression of sGC subunits have also been studied under conditions of low NO availability. Although animals in which NO production was impaired either by NO synthase gene knock-out (Brandes RP, 2000) or NO synthase inhibitor treatment (Rothermund L, 2000) showed an enhanced sensitivity towards vasodilator activity of exogenous NO, aortic sGC content was not altered as compared to wt mice (Brandes RP, 2000).

Cyclic AMP has consistently been found to down-regulate sGC levels in several tissues (Shimouchi A, 1993);(Papapetropoulos A, 1995);(Papapetropoulos A, 1996b). In some of these studies the downregulation of sGC expression was blocked by the inhibition of PKA (Papapetropoulos A, 1996b). In cases were inhibition of PKA was unsuccessful cross talk between cAMP and cGMP pathways resulting in the elevation of cGMP might have accounted for decreased sGC protein. Since in some studies high concentrations of cyclic nucleotide analogues were used, the reciprocal modulation of endogenously produced cAMP and cGMP could not be excluded.

There has been evidence that in various cells cAMP-eliciting agonists decrease the expression of sGC mRNA and protein (Shimouchi A, 1993);(Papapetropoulos A, 1995) by a destabilization of the sGC mRNA. This effect is mimicked by activation of the cGMP-signaling pathway, e.g. application of NO donors, stimulation of particulate guanyl cyclase by atrial natriuretic factor, and stimulation of cGMP-dependent protein kinase (PKG) by the stable cGMP-

analogue 8-chlorophenylthio-cGMP (Ujiie K, 1994). There is a recent article on post-transcriptional regulation of the α-1 subunit of NO-sensitive sGC in rat aorta (Kloss S, 2003) from our laboratory. HuR (Human R, embryonic lethal abnormal visual [ELAV]-like RNA-binding protein) has been identified as a factor stabilizing sGC α-1 mRNA. YC-1-induced activation of GC decreased HuR expression, thereby inducing rapid degradation of sGC α-1 mRNA and lowering α-1 subunit expression as a negative feedback response.

In endothelial cells, however, where cGMP was without any effect on sGC expression, the cAMP-elevating agent forskolin was also able to decrease sGC protein levels, suggesting a more complex action mechanism (Ibarra et al., 2001).

Various additional factors seem to have an effect on sGC expression. The cytokine IL1-β and TNFα, can reduce the α-1 and β-1 expression in smooth muscle cells and brain cells (Pedraza CE, 2003);(Takata M, 2001). It was demonstrated in Wistar rats that dietary supplementation with the antioxidant vitamin E restores normal endothelial function, reduces vascular superoxide anion formation and increases the expression of the sGC in rats with heart failure (Bauersachs J, 2001). The treatment with the hormone 17β-estradiol reduced the α-1 and β-1 mRNA expression and protein level in the uterus of young mice (Krumenacker JS, 2001). In rat pheochromocytoma PC12 cells, nerve growth factor (NGF) led to a transcription- and translation-dependent decrease in sGC α-1 and β-1 subunit mRNA and protein levels (Liu H, 1997). In astroglial cells ß-amyloid peptides appear to reduce sGC protein (Baltrons MA, 2002).

It is conceivable that variation of sGC expression enables a cell to adapt to the amount of NO available under certain physiological or pathophysiological conditions. Nitrate-tolerance to vasodilation by nitroglycerin (NTG) is a pathological condition of reduced NO bioavailability, which commonly occurs following prolonged exposure to NTG. In support of this hypothesis ncreased

expression of both sGC subunits was detected in blood vessels from NTG-tolerant rats and rabbits (Mulsch A, 2001).

Ageing and hypertension lead to reduced expression of sGC (Bauersachs J, 1998);(Chen L, 2000);(Ruetten H, 1999). In agreement with this, impaired vasodilation in response to the NO donor sodium nitroprusside has been reported in hypertensive rats (Kloss S, 2000). High salt intake by spontaneously hypertensive rats (SHR) led to a reduced vascular sGC content in the aorta, which was paralleled by impairment of the vascular relaxation response to NO donors (Kagota S, 2001).

Reduced sGC β-1 and sGC β-2 mRNA levels have been found in kidneys of Dahl salt-sensitive rats (Gupta G, 1997). In contrast, an increase in sGC protein levels was observed in aortic tissue of hypercholesterolemic rabbits (Laber U, 2002), which was considered by the authors as a compensatory mechanism for reduced (probably via oxidative damage) NO-sensitive sGC activity. Similarly, increased sGC protein levels have been found in rat heart after myocardial infarction (Bauersachs J, 1999) and in rat lung under hypoxic condition (Li D, 1999).

It should be noted that in many cases the exact mechanisms of sGC protein reduction are unknown. Moreover, in many studies sGC mRNA and not protein levels have been used to determine sGC regulation. It is speculated that changes in translation or transcription rate or destabilization of mRNA might play an important role for regulation of the protein amount (Pyriochou A, 2005).

Another interesting but poorly investigated aspect in the regulation of sGC is protein-protein interaction. It is well known that this kind of interaction plays important role in regulation of different signalling proteins. An exciting example is NOS with more than 20 proteins regulating the enzyme (Nedvetsky et al., 2002). Heat shock protein 90 (Hsp90) was the first sGC interacting protein to be identified (Venema et al., 2001). The N-terminal region of the β-1 subunit

mediates binding of the heterodimeric form of sGC to Hsp90. Hsp90 binding to sGC probably regulates the pool of active enzyme by affecting the protein levels of the two subunits (Papapetropoulos A, 2005). Protein interaction between CCT (chaperonin containing t-complex polypeptide) subunit eta and the α-1β-1 isoform of sGC has also been reported. Findings suggest that CCTeta binds to sGC (Hanafy KA, 2004) and, in cooperation with some other factor, inhibits its activity by modifying the binding of NO to the heme group or the subsequent conformational changes. The multidomain protein AGAP1, the prototype of an ArfGAP protein with a GTPase-like domain, Ankyrin repeats, and a pleckstrin homology domain has been identified as another sGC interacting protein (Meurer S, 2004). AGAP1 binds through its carboxyl terminal portion to both the α-1 and β-1 subunits of sGC. It is possible that there is a potential role of AGAP1 in integrating signals from Arf, NO/cGMP, and tyrosine kinase signaling pathways. Recently Hsp70 has been identified as a novel sGC-interacting protein (Balashova N, 2005) that is responsible for the sGC-activating effect, probably in association with other factors or after covalent modification.

4 The mRNA stability regulating protein HuR

The growth and development of eukaryotic organisms require that gene expression is regulated. Typically, this regulation is considered to occur at the level of DNA (differential transcription) or protein (selective degradation). However, gene expression can also be regulated at the level of mRNA. Posttranscriptional gene regulation occurs through alterations in translational efficiency and in mRNA stability (Ross J., 1995). Over the course of the last 15 years, many different types of mRNA decay have been described which alter the stability of particular mRNAs in eukaryotes. Both the signals and proteins participating in these processes have been identified. Some act to degrade, whereas others selectively stabilize mRNA.

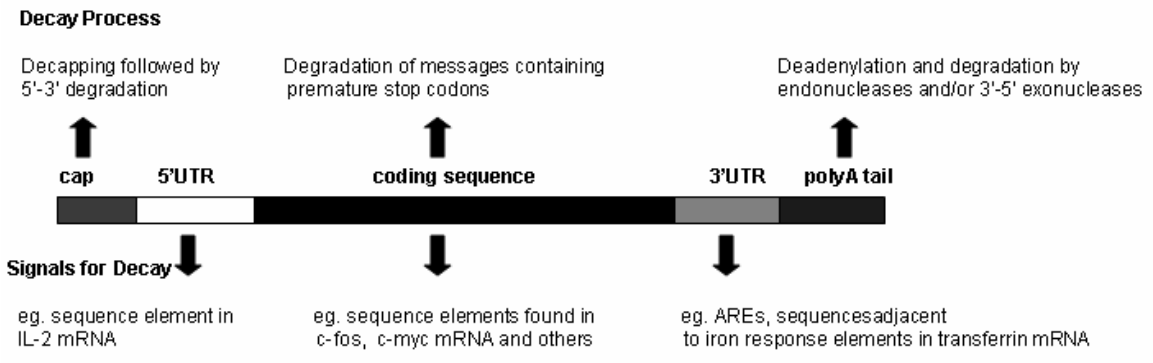


Figure 7. mRNA stability is determined by numerous cis-acting elements. Indicated above are decay processes that act on all mRNAs. Examples of decay signals found only in certain mRNAs are listed below.

3' untranslated regions UTRs often regulate decay. One well-studied example is the 3' UTR of transferrin mRNA, where an instability element is located adjacent to stem-loop structures called iron-responsive elements (IREs), which are reversibly bound by an iron-regulatory protein (IRP) leading to mRNA stabilization at reduced levels of cellular iron. The best-studied instability element in mammalian messages is the ARE (AU rich elements) (Chen CY, 1995). AREs consist of multiple stretches of adenylate and uridylate residues and are present in the 3' UTRs of many mRNAs, including those of growth factors, cytokines and lymphokines. Because of the importance of proteins encoded by ARE-containing mRNAs to normal and neoplastic cell growth, this element must be considered a pivotal gene regulatory target in vertebrate cells.

Class I AREs, such as the c-*fos* ARE, contain one to three scattered copies of the pentamer AUUUA embedded within U-rich regions (Wilson T, 1988). Class II AREs, like the GM-CSF ARE, consist of at least two overlapping copies of a critical nonamer UUAUUUA(U:A)(U:A) also in the context of a U-rich region (Lagnado CA, 1994). Class III AREs, an example of which is the c-*jun* ARE, lack the hallmark AUUUA pentamer but signal degradation with U-rich (and possibly other unknown) sequences. mRNAs containing class III AREs, like those containing class I AREs, exhibit degradation intermediates with 30–60 nucleotides of their poly(A) tail remaining.

In an effort to understand the mechanism and regulation of ARE-signaled mRNA degradation, a number of laboratories have looked for proteins that selectively bind AU-and U-rich sequences. At least 14, apparently distinct proteins have been identified in cell extracts by ultraviolet (UV)-crosslinking and gel-shift assays: AUBF (Malter JS, 1991), AU-A (Bohjanen PR, 1991), AU-B (Bohjanen PR, 1991), AU-C (Bohjanen PR, 1992), Hel-N1 (Levine TD, 1993), hnRNP D (Zhang W, 1993), hnRNP A1 (Hamilton BJ, 1993), hnRNP C (Hamilton BJ, 1993), AUH (Nakagawa J, 1995), GAPDH (Nagy E, 1995), hnRNP A0 (Myer VE, 1995), HuR (Ma WJ, 1996;Myer VE, 1997), tristetraprolin (Carballo E, 1998) and TIAR (Gueydan C, 1999). However, only two of these proteins, hnRNP D and HuR, have been demonstrated to alter the stability of ARE-containing mRNA in vivo.

RNAs bound by HuR:

RNA	ARE class
B-adrenergic receptor	III
Cyclin A	I
Cyclin B1	I
Cyclin D1	I
c-fos	I
c-myc	I
HPV-16 late	III
HPV-1 late	I
Herpesvirus saimiri-encoded U RNA-1	II
Herpesvirus saimiri-encoded U RNA-2	I
Herpesvirus saimiri-encoded U RNA-5	I
IL-3	II
N-myc	III
Neurofibromin	III
p21	I
Plasminogen activator inhibitor	I
Tumor necrosis factor-a	II
Vascular endothelial growth factor	III
GAP-43	III

HuR (or HuA) is a ubiquitously expressed member of the embryonic lethal abnormal vision (ELAV) family of RNA-binding proteins, (Ma WJ, 1996) originally

identified in *Drosophila melanogaster* as essential for neural development (Campos AR, 1985). Both gel shift and UV-crosslinking experiments have provided evidence that HuR binding parallels the in vivo ability of ARE sequences to direct mRNA degradation. However, overexpression of this protein does not enhance degradation, but rather stabilizes messages containing class I and class II (and to a lesser extent class III) AREs in transient transfection experiments (Fan XC, 1998a;Peng SS, 1998). Accordingly, most of the specific mRNAs bound by HuR in vitro contain class I or class II AREs. HuR appears to act by protecting the body of the message from degradation, rather than slowing the rate of deadenylation, in overexpressing cells (Peng SS, 1998). An alternative explanation for the stabilization observed in cells overexpressing HuR is that it is active in decay, but when overexpressed sequesters other factors needed for degradation. A HuR gene knockout should clarify this question. The finding that expression of antisense RNA to HuR increases the decay of ARE-containing mRNAs, (Wang W, 2000) is consistent with HuR's major role being to stabilize.

Among other RNAs, there has been recent evidence that the expression of sGC is subject to post-transcriptional regulation by HuR (Kloss S, 2003). It has been shown that cGMP- and cAMP-eliciting agonists decrease HuR expression in rat aortic tissue and cultured rat aortic smooth muscle cells and, at the same time, decrease expression of sGC α-1 and sGC β-1 subunits. These findings indicate that HuR is not only an important factor controlling vascular gene expression, but is also subject to control by vasoactive factors that regulate cGMP and cAMP levels (Kloss S, 2003);(Kloss S, 2004). The findings suggest that chronic hypertension induces changes in HuR expression and activity, which account for decreased sGC expression and activity in the aorta of hypertensive rats (Kloss S, 2005).

Like other Hu-family proteins, HuR contains three classic RNA recognition motifs (RRMs). At least in the case of HuD, ARE recognition appears to be mediated by

the first two RRMs; the third RRM of HuD has been suggested to bind the poly(A) tail. In transient transfection assays, deletion of RRM3 alone abolishes HuR's ability to stabilize ARE-containing reporter mRNAs (Fan XC, 1998a). Although predominantly nuclear, HuR shuttles between the nucleus and the cytoplasm via a novel shuttling sequence, HNS (HuR Nucleocytoplasmic Shuttling), located in the hinge region between its second and third RRM (Fan XC, 1998a);(Fan XC, 1998b). HNS is similar to the M9 shuttling sequence of hnRNP A1 but differs in several critical residues. The nuclear export receptor for HNS is not yet known. HuR's ability to shuttle has led to the suggestion that HuR may initially bind mRNAs in the nucleus and accompany them into the cytoplasm, providing ongoing protection from the degradation machinery. Recent in vivo crosslinking experiments and gradient analyses established that HuR is capable of binding poly(A)+ RNA in both the nucleus and the cytoplasm (Gallouzi IE, 2000). These data are consistent with the observation that a substantial fraction of cytoplasmic HuR (~15%) is associated with polysomes (Gallouzi IE, 2000).

In an effort to understand the intracellular interactions that regulate the activity of HuR in stabilizing ARE containing mRNA, several protein ligands to HuR have been identified and characterized (Brennan CM, 2000). They are SETa:b (Matsumoto K, 1993);(von Lindern M, 1992), pp32 (Malek SN, 1990) and acidic protein rich in leucine (APRIL) (Mencinger M, 1998). Three of these HuR ligands (SETa, SETb and pp32) had previously been identified as inhibitors of protein phosphatase 2A (PP2A) (Li M, 1996);(Saito S, 1999). PP2A is a multimeric serine:threonine phosphatase, affecting a diverse set of cellular functions including: cell cycle progression, DNA replication, transcription, splicing, development and morphogenesis (Millward TA, 1999). Deletion experiments suggested that the acidic tail of at least pp32 (and possibly these other HuR ligands) is required for their interaction with a region spanning the hinge region and third RRM of HuR (Brennan CM, 2000). Studies provide evidence for the in vivo interaction of HuR with its ligands and strongly suggest that these HuR

ligands either increase HuR's affinity for its target mRNAs or modulate HuR export from the nucleus.

II Materials & Methods

1 Materials

1.1 Cell Culture Reagents

Ham's nutrient media was purchased from PAA Laboratories GmbH, Linz, Austria. Dulbecco's Modified Eagle Medium (DMEM), Minimal Essential Medium (MEM), foetal calf serum (FCS), penicillin-streptomycin, and trypsin/EDTA were from GIBCO, Invitrogen Corporation, Grand Island, USA. Dulbecco's phosphate buffer saline (PBS) and Hank's buffered salt solution (HBSS) were purchased from GIBCO BRL, Life Technologies Limited, Paisley, Scotland. Foetal bovine serum was purchased from Biochrom KG, Berlin, Germany. Tissue culture flasks and dishes were from Nunc, Roskilde, Denmark. Disposable pipettes were purchased from Corning Incorporated, New York, USA. Bovine serum albumin (BSA) was from Sigma, Deisenhofen, Germany.

1.2 Molecular Biology Reagents

Effectene transfection reagent, QIAprep Spin miniprep kit, QIAGEN plasmid midi kit, PCR purification kit and gel extraction kit were purchased from QIAGEN, Hilden, Germany. Hybond nylon membrane and Taq DNA polymerase were purchased from Amersham Biosciences, Uppsala, Sweden. Electrophoresis grade agarose was purchased from GIBCO BRL Life Technologies Limited, Paisley, Scotland. Trizol and Superscript II Rnase H- reverse transcriptase were purchased from Invitrogen Life Technologies, Carlsbad, USA. Diethyl pyrocarbonate (DEPC) was purchased from Applichem, Darmstadt, Germany. The restriction endonucleases and T4 DNA ligase used were from New England Biolabs Incorporated, Beverly, USA. Pfu Turbo DNA polymerase was purchased from Stratagene, La Jolla, USA. Deoxynucleotides (dNTPs) were purchased from Carl Roth GmbH, Karlsruhe, Germany.

The following plasmids were used in this thesis:

pGL3-Basic

The pGL3 luciferase reporter vectors provide a basis for the quantitative analysis of factors that potentially regulate mammalian gene expression. These factors may be cis-acting, such as promoters and enhancers, or trans-acting, such as various DNA-binding factors. The backbone of the pGL3 luciferase reporter vectors contains a modified coding region for firefly (*Photinus pyralis*) luciferase that has been optimized for monitoring transcriptional activity in transfected eukaryotic cells.

pRL-TK

The pRL-TK vector is intended for use as an internal control reporter and may be used in combination with any experimental reporter vector to cotransfect mammalian cells. It contains a cDNA (Rluc) encoding renilla luciferase, which was originally cloned from the marine organism renilla reniformis. The pRL-TK vector contains the herpes simplex virus thymidine kinase (HSV-TK) promoter to provide low to moderate levels of renilla luciferase expression in cotransfected mammalian cells.

pLexA

pLexA is used to generate fusions of the DNA binding domain with a target (or bait) protein. Fusion protein expression is controlled by the strong yeast ADH1 (alcohol dehydrogenase) promoter. It can be propagated and selected for in *E. coli* and yeast. The HIS3 (Histidine) transformation marker is used for selection in yeast.

pJG4-5

pJG4-5 expresses cDNAs or other coding sequences inserted into the unique EcoRI and XhoI sites as translational fusions to a cassette consisting of the SV40 (Simian Virus 40) nuclear localization sequence, the 88 residue acidic activator

(activation domain), and the hemagglutinin epitope tag for the construction of fusion proteins. Fusion protein expression is under the control of the GAL1 (galactose) inducible promoter. It can be propagated and selected for in *E. coli* and yeast. The TRP1 (tryptophan) transformation marker is used for selection in yeast.

pSHI8-34

pSHI8-34 carries the lacZ reporter gene under the control of eight LexA operators and the minimal TATA region from the GAL1 promoter. pSHI8-34 exhibits zero transcriptional activity in the absence of a LexA-fused activator. pSHI8-34 can be maintained as an autonomously replicating plasmid in strain EGY48 or can be forced to integrate into the EGY48 genome.

pGEX

GST (glutathione-S-transferase) Gene Fusion System incorporates pGEX plasmids for inducible, high-level intracellular expression of genes or gene-fragments as fusions with *Schistosoma japonicum* GST. Expression in *E. coli* yields fusion proteins in the cytoplasm with the GST moiety at the amino terminus and the protein of interest at the carboxyl terminus.

pBSKS (BlueScript)

Some useful features of pBluescript: there is a multiple cloning site inserted into the LacZ gene, so clones with inserts can be distinguished as blue versus white cells by staining using X-gal (5-bromo-4-chloro-3-indolyl-bD-galactoside). It has a T3 promoter on one end side and a T7 promoter on the other terminal end. Other features are 21 unique restriction sites in the multiple cloning region, blue/white color selection and double and single-stranded sequencing.

The following *E.coli* strains were used:

BL21

F¨C ompT hsdS(rB¨C mB¨C) dcm+ Tet^{r} gal endA Hte [argU ileY leuW Cam^{r}]

The BL21 series of *E. coli* hosts are often the first choice for protein expression. They are protease deficient and designed for high-level protein expression from T7 RNA polymerase-based expression systems. Derived from *E. coli* B, these strains naturally lack the Lon protease and are engineered to be deficient for the OmpT protease. The Lon and OmpT proteases found in other *E. coli* expression hosts may interfere with the isolation of intact recombinant proteins.

DH5α

F- ϕ80dlacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ- thi-1 gyrA96 relA1

DH5α is a well-known, versatile strain that can be used in many everyday-cloning applications. In addition to supporting blue/white screening, recA1 and endA1 mutations in DH5α increase insert stability and improve the quality of plasmid DNA prepared from minipreps.

HB101

F–, thi-1, hsdS20 (rB–, mB–), supE44, recA13, ara-14, leuB6, proA2, lacY1, galK2, rpsL20 (strr), xyl-5, mtl-1

HB101 cells are useful for cloning in vectors that do not require complementation for blue/white screening. The competent cells can be used for many standard molecular biology applications.

1.3 Biochemical Reagents

Acrylamide/bisacrylamide solution, ammonium persulphate, coomassie stain, ponceau-s stain, dithiothreitol, glutathione, β-mercaptoethanol, isopropylthiogalactoside, leupeptin, aprotinin, pepstatin, Triton X-100 and Tween-20 were from Sigma, Diesenhofen, Germany. Molecular weight markers, Roti load 4X were from Roth, Karlsruhe, Germany. Sodium dodecyl sulphate and glycerol were from Merck, Darmstadt, Germany.

1.4 Animal Species and Maintenance

Male Wistar rats (300-400 gm)(Harlan Winkelmann, Borchen, Germany) were used. The animals were kept in 550x330x200 mm sized Makrolon cages (Erich Becker, Germany) on dust-free wood granules (Altromin, Germany). They were fed with the Altromin-Standard-Diet No. 1320 and tap water ad libitum. The ambient temperature of the animal room was between 21° - 25° C with a relative air humidity of 45 - 65 %. The area was lit up with neon lamps in a circadian light-dark rhythm in the ratio 10:14 (7.00 – 17.00 o'clock).

1.5 Cell Lines

RLF-6 cells (rat lung fibroblast cell line) were a kind gift from Prof. U. Foerstermann, Institute of Pharmacology, Mainz University. They were maintained in Ham's nutrient medium F12 supplemented with 15 % foetal calf serum (FCS).

COS-1 cells (monkey kidney cell line) were a kind gift from Dr. Stephen Gross, Institute of Biochemistry, Frankfurt University, and were cultured in DMEM and 10 % FCS.

Primary cultures of rat aortic smooth muscle cells were obtained as described below and were maintained in MEM with 10 % FCS and 1 % penicillin-streptomycin.

1.6 Buffers and Media

Media for Bacteria

Luria-Bertani Medium (LB)

grams per Liter	Constituents
10	Bacto-Tryptone
5	Bacto-Yeast Extract
10	NaCl

20ug of Ampicillin per ml of LB medium for LB-Ampicillin medium

Media for Yeast

Yeast Peptone Dextrose Medium (YPD)

grams per Liter	Constituents
10	Yeast Extract
20	Peptone
20	Dextrose

SD/–Ura, SD/–His, SD/–Trp/–Ura, SD/–His/–Trp/–Ura, SD/–His/–Ura Medium

per Liter	Constituents
6.8	Yeast Nitrogen Base
300 ul	12.5 M NaOH
2%	Glucose
700 mg	DO Supplement(–Ura, –His, –Trp/–Ura, –His/–Trp/–Ura, – His/–Ura)

SD/Gal/Raf/–His/–Trp/–Ura, SD/Gal/Raf/–His/–Leu/–Trp/–Ura Medium

per Liter	Constituents
6.8	Yeast Nitrogen Base
300 ul	12.5 M NaOH
2%	Galactose
1%	Raffinose
80 ul	X-Gal
2 ml	BU Salt
700 mg	DO Supplement(–Ura, –His, –Trp/–Ura, –His/–Trp/–Ura, –His/-Ura)

Stocks for Yeast Media

BU Salt

per Liter	Constituents
70 g	$Na_2HPO_4 \cdot 7H_2O$
30 g	NaH_2PO_4

X-Gal

	Constituents
1 ml	N,N-dimethylformamide (DMF)
20 g	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal)

Buffers

4x Laemmli-buffer

per Liter	**Constituents**
125 mM	Tris/HCl pH 6.8
10% (w/v)	SDS
50 mM	Dithiothreitol
30% (v/v)	Glycerol
0.01% (w/v)	Bromphenol blue

10x PAGE

per Liter	**Constituents**
250 mM	Tris
1% (w/v)	SDS
520 mM	Glycine

50x TBST

per Liter	**Constituents**
0.1 M	Tris pH 8.0
1.5 M	NaCl
5% (v/v)	Tween 20

Transfer buffer

per Liter	**Constituents**
25 mM	Tris
192 mM	Glycine pH 8.3
20% (v/v)	Methanol

Additional buffers and solutions

10x PBS (pH 7.4)

per Liter	Constituents
1.3 M	NaCl
30 mM	NaH_2PO_4
70 mM	Na_2HPO_4

50x TAE

per Liter	Constituents
2 M	Tris
1 M	Acetic acid
50 mM	EDTA

10x TBE

per Liter	Constituents
0.45 M	Tris
0.45 M	Boric acid
10 M	EDTA

1.7 Laboratory Equipments

Gel dryer 583, Gel Doc 1000; Bio-Rad, Germany

T Gradient Thermocycler, UV Photometer Gene Ray, Shaker Heater Incubator (Compact Line OV); Biometra, Germany

Herasafe Clean Bench, Incubator Heraeus BBD 6220, Centrifuge Biofuge; Heraeus, Germany

Sonicator; Gerhard Heinemann Labor, Germany

Vortex Genie2; Scientific Industries, USA

Test tube thermostat; Roth, Germany

Shaker GFL 3005; Orbital, Germany

Luminometer LB953; EG & G Berthold,Germany

Light Microscope; Carl-Zeiss, Germany

1.8 Computer Programmes

Microsoft Office 2000, Reference Manager 9, Adobe V, I-Photoshop 6, Scion Image 4.02, Corel Draw 5, were the softwares used.

NCBI-Blast
(http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed&itool=toolbar)
and Matinspector V2.2
(http://www.genomatix.de/?s=78d4cce024079eadfd01775177277a4c)
were used on the Internet.

1.9 Statistical calculations

Statistical calculations were performed using GraphPad Prism 3.0 statistical package (GraphPad Software, San Diego, California, USA). Statistical differences between the means were analysed by Student's unpaired t-test. For multiple comparisons, one-way analysis of variance (ANOVA) test was employed.

2 Methods

2.1 Nucleic Acid Preparation

2.1.1 Plasmid Preparation

The method employed for plasmid preparation was dictated by the purity and quantity of DNA required.

2.1.1.1 Small-scale Plasmid Preparation from E.coli

2 ml LB was inoculated with a single colony of the respective *E. coli* clone and incubated for 8-16 h at 37°C at 160 rpm. One ml each of the grown cultures was taken in a 1.5 ml eppendorf tube and centrifuged for 30 seconds at 10.000xg. The pellet was resuspended in 100 µl TE (50/10)/RNaseA (10 mg/ml in 50 mM potassium acetate pH 4.8, heat-inactivated) and cells were lysed with 200 µl of 0.2 M NaOH/1% SDS. 200 µl of 3 M potassium acetate was added and centrifuged for 5 minutes at 10,000xg. The supernatant was transferred to a fresh tube, 500 µl isopropanol was added and centrifuged for 10 minutes at 10.000xg at 4°C. The resulting pellet was washed with 500 µl of 70% ethanol, centrifuged again for 5 minutes, air dried and dissolved in 20-50 µl of sterile water.
High quality mini plasmid preparation was performed using the QIAGEN Plasmid Mini Kit. The protocol used was identical to the manufacturer's instructions

2.1.1.2 Large Scale Plasmid DNA Preparation from E.coli

E.coli DH5α cells were transformed as described above. An individual colony was picked up from the LB plates or 50 µl aliquot was taken from glycerol stock and was inoculated in 5 ml LB medium (incubation overnight, 37°C, 160 rpm (Infors, Einbach, Germany). 100 -150 µl of this preculture was inoculated into a large culture with 50 ml LB-ampicillin medium (incubation overnight, 37°C, 160 rpm). The culture was transferred in centrifuge tubes and pelleted at 6000xg, 30 minutes and the plasmid DNA was prepared using the Qiagen Midi Plasmid Purification kit. Subsequently, the DNA was precipitated with 0.7 volumes of isopropanol and centrifuged at 15000xg for 30 minutes at 4°C. After air-drying

the pellet, it was dissolved in 400 µl of sterile water, transferred into a 1.5 ml eppendorf tube and precipitated again by the addition of 2 volumes of 100 % ethanol, 1/10 volume of sodium acetate (pH 5.2) and 30 minutes incubation at -20°C. After centrifugation (30 minutes, 12000xg, 4°C), the pellet was washed with 70 % ethanol (5 minutes, 12000xg, 4°C), air-dried, and redissolved in an appropriate volume of sterile water.The DNA yield was determined with the help of the UV spectrophotometer. The optical density (OD) was measured at 260 and 280 nm (Biometra, Gottingen, Germany). As a check for impurities the quotient of the OD260/OD280 was also calculated, which should lie between 1.8 and 2.0 (empirically found value). The plasmid DNA was then examined by running on a 1% agarose gel.

2.1.1.3 Small Scale Plasmid Preparation From Yeast

Five ml of an overnight culture of yeast growing in the specific selection medium was sedimented by centrifuging at 4000 rpm for 10 minutes. After decanting the supernatant, the pellet was resuspended in 30 µl of lyticase solution (10 mM Tris-HCl pH 8, 1 mM EDTA, 4.5 units/ml Lyticase – filter-sterilised and stored at 4^0C) and incubated at 37^0C for 1 hour. 170 µl of the lysis buffer (10 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM EDTA, 2% Triton X-100, 1% SDS) was then added with brief vortexing. 200 µl glass-beads and 200 µl of phenol/chloroform mixture was added to the suspension and vortexed for 5 minutes at maximum speed. The tubes were then centrifuged at 13,000 rpm for 10 minutes. The top aqueous phase was transferred to a fresh eppendorf tube and the DNA precipitated with 10 M ammonium acetate and 100 % ethanol. The tube was placed at -20^0C for at least 1 hour and then centrifuged at 13000 rpm for 10 minutes. The pellet was washed with 70% ethanol, air dried and dissolved in 20 ml H_2O.

2.1.2 RNA Preparation

Due to the susceptibility of RNA to degradation by RNAses all solutions used were prepared with diethylpyrocarbonate (DEPC) water. DEPC treated water was obtained by adding 1 ml DEPC per litre of distilled water, incubating overnight at RT and then autoclaving it.

RNA isolation was performed with Trizol reagent according to the manufacture's instructions. Cells were grown and stimulated as described above. A pipette tip attached to a vacuum line removed last traces of media. Subsequently, cells were lysed with 1 ml of Trizol per 3 wells using 6-well-plates. In case of using isolated rat aorta, the tissue was frozen after stimulation and then homogenised in a tissue homogenator (B. Braun, Melsungen, Germany). 1 ml of the Trizol solution was added to every 100 mg tissue. The lysate was transferred into an eppendorf tube. After addition of 200 µl chloroform, the samples were inverted for 30 seconds. The inverted tubes were stored at RT for 10 minutes and subsequently centrifuged (11000 rpm, 10 minutes at 4°C). Afterwards the aqueous upper phase was transferred into a fresh tube. RNA was precipitated using 500 µl of isopropanol and stored again for 10 minutes at RT. Finally a single centrifugation step was carried out at 13000 rpm for 15 minutes at 4°C to pellet the precipitated RNA. Then the RNA pellet was washed with cold 70% ethanol (with DEPC-treated water), followed by centrifugation (13000 rpm, 10 minutes at 4°C). The final RNA pellet was resuspended in 20 – 25 µl DEPC-treated water. After 10 minutes incubation at 55°C, the amount of isolated RNA was quantified photometrically.

2.1.3 cDNA Synthesis

First strand cDNA synthesis was performed by incubating the following at 65^0C for 5 minutes in a nuclease free tube: 1 µl oligo (dT), 2 µg total RNA, 1 µl 10 mM dNTPs and MQ-H_2O to 12 µl. The reaction mix was quickly chilled on ice before the addition of 4 µl 5X first strand buffer, 2 µl 0.1 M DTT and 1 µl RNAse out (40

U/μl). The reaction mix was then incubated at 42^0C for 2 minutes before the addition of 1 μl (200U) of Superscript II. Incubation at 42^0C was carried out for a further 50 minutes followed by a 15-minute incubation at 70^0C (Biometra, Gottingen, Germany). The cDNA was then stored at -20^0C till required.

2.2 Nucleic Acid Quantitation

The concentration of DNA and RNA samples was determined by spectrophotometry at 260 nm (Biometra, Gottingen, Germany). Each OD unit at 260nm corresponds to 50 μg/ml of double stranded DNA and 40 μg/ml of RNA (Sambrook et al. 1989).

2.3 Restriction endonuclease digestion of DNA

The digestion of DNA using the restriction enzymes was carried out in accordance with the manufacturer's instructions. The standard reaction mix consisted of: DNA template, 10x stock buffer with a final concentration of 1x in the reaction mix, 1-10 units of the enzyme depending on the amount of DNA to be digested and H_2O. These mixtures were incubated in a water bath at 37^0C for 1-6 hours.

2.4 Agarose gel electrophoresis of DNA

The DNA samples were mixed with 1/6 volume of the DNA loading buffer (6x: 50% (v/v) glycerol, 0.01% (w/v) bromphenolblue, 0.01% (w/v) xyleneblue) and resolved in horizontal agarose gel electrophoresis at 40-120 V. The agarose concentration in the gel depended upon the size of the fragments to be separated [0.8-2% in 1x TAE (w/v)]. 1x TAE (40 mM Tris acetate, 1 mM EDTA) was used as the electrophoresis buffer. The gels were then stained in 0.1% ethidium bromide (EtBr) solution for 5 minutes and photographed under UV light (Fujifilm, Amersham Pharmacia).

2.5 SDS Gel Electrophoresis

Electrophoretic separation of proteins was carried out in the discontinuous buffer system for SDS polyacrylamide gels as originally described by Laemmli (1970). It was carried out using the Mini-Gel systems (Pharmacia Biotech, Uppsala, Sweden). Preparation of 8-15% separating gel and 5% stacking gel was done as following. 20 - 40 µg of total protein were diluted in 4x Roti Load1, boiled for 5 minutes and loaded onto the gel. Prestained molecular weight markers were also loaded on each gel. Electrophoresis was performed at 100 V until the samples had reached the separating gel after which it was increased to 200 V.

Table 4: List of the composition of upper and lower gels in SDS electrophoresis.

10x SDS Laemmli Running Buffer (0.25 M Tris, 1.92 M Glycine, 1% SDS, pH 8.3)

Lower Gel: 4x Laemmli Lower Tris (1.5 M Tis, 0.4% SDS, pH 8.8)

% Total Acrylamide:		8%	10%	12%	15%
H_2O	(ml)	12.1	10.5	8.75	6.25
4x Lower Tris Buffer	(ml)	6.25	6.25	6.25	6.25
29:1 Acrylamide:Bis	(ml)	6.67	8.35	10	12.5
10% APS	(l)	130	130	130	130
TEMED	(l)	14	14	14	14
Total Vol:		25 ml	25 ml	25 ml	25 ml

Upper Gel: 4x Laemmli Upper Tris (0.5 M Tris, 0.4 % SDS, pH 6.8)

% Total Acrylamide:		5%	5%
H_2O	(ml)	5.8	11.6
4x Upper Tris Buffer	(ml)	2.5	5
29:1 Acrylamide:Bis	(ml)	1.7	3.33
10% APS	(l)	40	60
TEMED	(l)	20	20
Total Vol:		10 ml	25 ml

2.6 Electrophoretic Mobility Shift Assay(EMSA)

Nuclear extracts from RLF-6 cells were prepared as described above. Binding was tested in 20 µl of solution by incubating 1-2 µg of nuclear extract with a biotinylated probe (MWG Biotech, Ebersberg, Germany) in reaction buffer {(10 mM Tris, 50 mM KCl, 10 mM DTT) pH 7.5, 2.5% glycerol, 5 mM $MgCl_2$} for 15 minutes at 4^{o}C. For supershift assays, specific antibodies (Santa Cruz Biotechnology, Santa Cruz, USA) were added to the EMSA binding reaction 15 minutes before the addition of probes and incubated on ice. The protein-DNA complexes were separated on a 8% non-denaturing polyacrylamide gel in ice-cold 1x TBE buffer for 3 hours at 80 V. Gels were transferred onto Hybond-N+ nylon membranes (Amersham Biosciences, Uppsala, Sweden) by placing in between 3 layers of gel blotting paper (Schleicher and Schuell, Dassel, Germany) prewetted in 1x TBE Buffer in a semi-dry electroblotter (PeqLab, Erlangen, Germany) for 2 hours at 150 mA. The DNA was cross-linked to the membrane for 10-15 minutes with the membrane face down on a transilluminator equipped with 312 nm bulbs. After drying, the detection of the biotin labelled DNA was carried out using the LightShift Chemiluminescent EMSA kit (Pierce Biotechnology, Rockford, USA) according to the manufacturer's protocol. Briefly, the membrane was incubated in the LightShift blocking buffer for 15 minutes in a clean tray on a shaker after which the buffer was replaced with the LightShift stabilised streptavidin-horseradish peroxidase conjugate diluted in the blocking buffer in a ratio of 1:300 and incubated for another 15 minutes. The membrane was washed 5 times for 5 minutes each with 1x wash buffer and incubated in the LightShift substrate equilibration buffer for 5 minutes. The LightShift luminol/enhancer solution and the LightShift stable peroxide solution were mixed in a ratio of 1:1 and added on to the membrane for 5 minutes after which the membrane was kept in a plastic wrap and excess solution was removed. The membrane was then placed in a film cassette and exposed to X-ray film.

2.7 DNA extraction from agarose gels

DNA bands were visualised on a UV transilluminator, excised with a sterile scalpel, weighed, and then isolated from the gel using the QIAGEN gel extraction kit according to the manufacturer's instructions.

2.8 Amplification of DNA using the Polymerase chain reaction (PCR)

PCR was used to amplify segments of DNA and was carried out in 0.2 ml thin-walled PCR tubes. A typical 25 μl reaction mixture consisted of: 10-500 ng of template DNA, 10 pmol each of forward and reverse primers, 200 μM dNTPs, 1-2 mM $MgCl_2$, and H_2O. Just prior to thermal cycling, 0.2 μl of the Taq DNA polymerase was added to the mix. Amplification was carried out in a T-gradient thermocycler. After the completion of the PCR cycles, products were electrophoresed on an agarose gel to determine the efficiency of amplification.

2.8.1 Primer Design

Primers were produced by MWG Biotech, Ebersberg, Germany. The software indicated in Table 5 was used to check for specificity of the primers.

Table 5: List of primers used for α-1 and β-1 sGC sequencing.

Name	5´---- 3´ Sequence	Position
α1-sGC Forward	GAAATCTTCAAGGGTTATG	1527 – 1545*
α1-sGC Reverse	CACAAAGCCAGGACAGTC	2352 – 2335*
β1-sGC Forward	GGTTTGCCAGAACCTTGTATCCACC	1491– 1515+
β1-sGC Reverse	GAGTTTTCTGGGGACATGAGACACC	1775 – 1750+
eloII- Forward	GACATCACCAAGGGTGTGCAG	2039 - 2059†
eloII- Reverse	GCAGTCAGCACACTGGCATA	2257 - 2256†

* GenBank®/EMBL Accession Number: M57405
\+ GenBank®/EMBL Accession Number: M22562
† GenBank®/EMBL Accession Number: NM017245

2.8.2 Thermal Cycling Programme

A basic thermal cycling program was employed for all PCRs conducted in this thesis. Firstly an initial denaturation step was carried out at 94^0C for 5 minutes. This was followed by 30-40 cycles of denaturation at 94^0C for 30 seconds, annealing at 50-60^0C for 45 seconds depending on the primers used and elongation at 72^0C for 30-120 seconds dictated by the length of DNA to be amplified. A final elongation step was carried out at 72^0C for 15 minutes. The PCR products were electrophoresed on an agarose gel and purified as mentioned above.

2.9 Ligation of DNA

A typical ligation mix consisted of 25-200 ng of the vector DNA, an appropriate amount of the insert DNA, 1x ligase buffer, 1 μl of the T4 DNA ligase and sterile H_2O in a total volume of 10 μl. This mix was incubated in a water bath at 4^0C overnight or at room temperature for 2 hours and subsequently used to transform competent *E.coli* cells.

2.10 Preparation of Plasmid Vector

In general, plasmids used for cloning were linearised with appropriate restriction enzymes, and dephosphorylated if compatible ends were produced. Dephosphorylation involved the addition of 2 μl of calf intestinal alkaline phosphatase (CIAP 1:40 in 1x phosphatase buffer), 6 μl phosphatase buffer and 2 μl sterile MQ-H_2O to a 50 μl plasmid digest. The reaction was incubated at 37^0C for 30 minutes and 85^0C for 15 minutes. The dephosphorylated linearised plasmid was then analysed by gel electrophoresis and purified by gel extraction.

2.11 Transformation of E.coli cells

2.11.1 Preparation of competent *E.coli* cells

100 ml of LB medium was inoculated with an overnight culture of *E.coli* DH5α cells in a ratio of 1:100 and placed in a shaker-incubator at 37°C. When the OD_{600} reached between 0.4 and 0.6, the cells were centrifuged (10 minutes, 1500 g, 4°C). Subsequently, the cells were resuspended in 20 ml RF1 buffer (100 mM RbCl, 50 mM $MgCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$, 15% glycerin, pH 5.8) and kept on ice for 2 hours. After sedimentation of the cells by centrifugation, 4 ml RF2 buffer (10 mM MOPS, 10 mM RbCl, 75 mm $CaCl_2$, 15% glycerin, pH 6.8) was added and incubated for 15 minutes on ice. This suspension of competent *E. coli* cells was kept in -70°C in 500 µl aliquots.

2.11.2 Transformation of competent *E. coli*

To 150 µl competent DH5α cells, approximately 1 µg plasmid DNA was added and kept on ice for 30 minutes. Heat shock was given by placing the mix in 42°C water bath for 2 minutes, after which 600 µl of LB medium was added and incubated for 1 hour at 37°C. 100 - 150 µl of the cell suspension was plated on LB Ampicillin plates and incubated overnight in the incubator at 37°C.

2.12 Bacterial frozen stocks

Frozen bacterial stocks of DNA clones were made by inoculating a single colony in 3 ml of LB medium containing the specific antibiotic. The culture was incubated overnight at 37°C with shaking. Equal volumes of bacterial culture and 50% sterile glycerol were then mixed and frozen immediately at -70°C.

2.13 Transformation of yeast

A single yeast colony containing the appropriate plasmid was inoculated in 5 ml of the specific selective medium and incubated overnight at 30°C at 200 rpm. 10 ml of YPD medium was inoculated with this overnight culture to an OD_{600} of 0.1-0.2 and incubated at 30^0C with shaking for 3-5 hours until the OD_{600} reached 0.4-0.8. 2.5 ml of the culture was harvested by centrifuging at 4000 rpm for 5 minutes. The pellet was washed with 0.5 volumes of sterile H_2O and centrifuged same as above. To the cell pellet was added in the following order: 45 μl sterile H_2O, 240 μl 50% PEG-4000, 36 μl 1 M lithium acetate, 5 μl carrier DNA, 1-5 μg plasmid DNA. The suspension was vortexed for at least 1 minute and incubated at 30^0C for 30 minutes. Heat shock was performed by placing at 42^0C for 30 minutes with mixing after every 10 minutes. The cells were collected by centrifuging and the resulting pellet was resuspended in 100 - 200 μl of sterile H_2O, plated on the appropriate selection plates and incubated for 2 - 4 days at 30^0C.

2.14 Yeast mating assay

Single yeast colonies from opposite mating strains were inoculated in 5 ml of the specific selective medium and incubated overnight at 30^0C at 200 rpm. A 1:5 dilution of the cultures was prepared in YPD medium. For the mating, 250 μl each from both the yeast strains were mixed and incubated overnight at 30^0C. 10 μl from each mating was placed on appropriate selection plates and incubated for 2-4 days at 30^0C.

2.15 Cell culture

2.15.1 Isolation of smooth muscle cells from rat aorta

Rat aortic smooth muscle cells were obtained in primary culture. Thoracic section of aorta was dissected from male Wistar rats and kept in ice-cold HBSS. The aorta was cut open and the endothelium was removed with a cotton swab. The adventitia was then removed with fine forceps under a light microscope (Carl Zeiss, Jena, Germany). Aorta was cut into small pieces (1 x 1 mm) and transferred to 2 ml HBSS in which 1 mg/ml BSA, 380 U/ml collagenase 4 and 0.9 U/ml elastase (Worthington Biochemical Corporation, Lakewood, USA) were added and incubated at 37°C for 30 minutes. Single cells were released by gentle triturating through a pasteur pipette. Cells were centrifuged at 800 rpm for 4 minutes, washed once with HBSS and resuspended in MEM with 10% foetal calf serum (FCS) and 1% penicillin-streptomycin. The presence of smooth muscle cell population was confirmed by staining with monoclonal mouse anti-α-smooth muscle actin–FITC conjugate antibody.

2.15.2 Cell lines and media

RLF-6 (fibroblast cell line) were a kind gift from Prof. U. Förstermann, Pharmakologisches Institut der Universität Mainz. They were maintained in Ham's Nutrient Medium F12 supplemented with 15% foetal calf serum.

COS-1 cells (monkey kidney cell line) were a kind gift from Dr. Stephen Gross and were cultured in Dulbecco's Modified Eagle Medium (DMEM) and 10% FCS.

2.15.3 Revival of frozen cells

Cell line stocks were kept frozen under liquid nitrogen for long term storage. To revive cells, a frozen aliquot was thawed with 5 ml of prewarmed media by placing some of the media on top of the frozen cells and then sucking it up with the help of a 5 ml pipette. This was done repeatedly till the aliquot fully thawed after which they were transferred in centrifuge tubes and another 5 ml of the

media was added. The cells were centrifuged at 1000 rpm for 4 minutes, the pellet was resuspended in 5 ml of media and the entire content was transferred to a 25 cm^2 flask and kept in a humidified incubator at 37^0C supplemented with 5% CO_2.

2.15.4 Passaging and maintaining cells

Passaging was carried out once the cells were confluent. Media, PBS and the trypsin-EDTA solution were prewarmed to 37^0C. For trypsinisation, media was aspirated from within the flask, the cells were washed with 5 ml PBS and the trypsin-EDTA solution was added (2 ml to 75 cm^2 flask or 1 ml to 10 cm petri-dish). They were then placed in the incubator at 37^0C for 2-5 minutes. The enzymatic reaction was then stopped by adding 5 ml of the media with FCS, the cells were then transferred to a sterile centrifuge tube. Cells were sedimented at 1000 rpm for 4 minutes, the pellet was resuspended in 10 ml of the media and then split in appropriate ratio in 75 cm^2 flasks or 10 cm petri-dishes. Fresh media changes were carried out every 2-3 days.

2.15.5 Freezing cells for storage

Cells were prepared for freezing by culturing to 80-90% confluency in a 75 cm^2 flask. Trypsinisation was carried out as mentioned. The pellet was resuspended in a specific volume of media. To this, an equal volume of 20% DMSO in FCS was added, mixed and then aliquoted into labelled cryotubes. These tubes were placed at -80^0C for at least 24 hours before placing them for long term storage under liquid nitrogen.

2.16 Transient transfection of RLF-6 cells

The Effectene method (Qiagen) was used for the transient transfection of plasmid DNA into RLF-6 cells. This method is based on the formation of micelles, which fuse with the cell membrane releasing the plasmid inside. 0.4 µg of the reporter constructs (firefly luciferase) was used for the transfection experiments. As a control for efficiency of transfection 0.1 µg of pTK renilla coding for renilla luciferase under the control of herpes virus promoter was also used. Cells were

seeded at a concentration of 200,000 cells per well in a six-well plate and transfected on the next day in accordance with the manufacturer's protocol. After approximately 16 hours, cells were washed with PBS and starved for a further 24 hours before stimulation. All transfections were performed in duplicate or triplicate.

2.17 Transient transfection of COS-1 cells

A modified and more simplified version of the DEAE-Dextran protocol was used for thr transfection of COS-1 cells. Cells were seeded at a concentration of 0.6 x 10^5/ml 16 - 20 hours prior to transfection. On the day of transfection, cells were washed with PBS and were incubated with solution I (1 - 3 μg of the DNA to be transfected, 50 μg/ml DEAE-Dextran, 50 μg/ml chloroquine in serum free medium) at 37^0C for 2 and a half hours. The solution was thereafter removed, cells were washed with serum free media and then incubated with solution II (10% DMSO in PBS) for 2 minutes at room temperature. This was then aspirated and cells were washed with serum free media. An appropriate volume of the media with serum was then added and the cells were kept in the incubator till harvest.

2.18 Decoy oligodeoxynucleotide (ODN) technique

Double stranded ODN were prepared from complemetary single-stranded phosphorothioate-bonded oligos (obtained from MWG-Biotech, Ebersberg, Germany) by melting at 95°C for 5 minutes followed by a cool-down phase of 3-4 hours at ambient temperature. 10 nM of the various decoy ODN were transfected along with the reporter and the renilla constructs where required.

2.19 Determination of Luciferase Activity

In the DLR (Dual Luciferase Reporter) assay system, the activities of the firefly and renilla luciferases are measured sequentially from a single lysate. Upon completing the measurement of firefly luciferase activity (the "experimental" reporter), the firefly luminescence is rapidly quenched, with simultaneous activation of the renilla luciferase luminescent reaction (the "control" reporter

activity). Thus, the DLR assay system integrates the two assay chemistries to provide rapid quantitation of both reporters co-expressed in the lysates of transfected cells.

The use of firefly (*Photicus pyralis*) luciferase as reporter gene involves the adenosine triphosphate (ATP) dependent oxidative decarboxylation of luciferin, producing light emission at a wavelength of 562 nm.

$$\text{Luciferin} + \text{ATP} * O_2 + Mg^{2+} \rightarrow \text{Oxyluciferin} + \text{AMP+PPi} + CO_2 + \text{Light}\uparrow$$

The light emitted achieves its highest intensity after approximately 0.3 seconds and fades away thereafter within few seconds completely. Therefore it is important to use a luminometer that injects the reaction-mix to the cell lysate and measures the quantitative luminiscence with high speed. 15 - 50 µl of the cell lysate was put in luminat tubes and placed in the luminometer. Thereafter the injection of the luciferase reaction-mix and the measurement of the light emission (relative light unit (RLU)) was performed by the luminometer. If the light emitted was outside the linear range, measurements were repeated with lesser cell lysate volume. After the first measurement, the solution was replaced with substrate to quantify the *Renilla reniformis* luciferase. This solution contained the necessary components for the renilla luciferase and blocked at the same time the activity of the firefly luciferase. This solution was then injected to the samples already measured and the RLU was determined.

2.20 Preparation of protein extracts for gelshift assays

The protein extraction protocol was adopted from the method as described by Schreiber et al. For the cytosolic extract, 200 µl of ice-cold Buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 5 µg/ml PIM) was added to approximately 10 mg of frozen tissue in 1.5 ml eppendorf tubes or 2 x 10^5 cells in culture dishes after washing once with ice-

cold PBS. These were left on ice for 30 minutes after which the cells were scraped with a cell-scraper (Greiner, Kremsmuenster, Austria) and transferred into 1.5 ml eppendorf tubes. 25 µl of 10% Igepal CA 30 was added to the suspension and vortexed for 10 seconds. The homogenate was centrifuged at 13,000 rpm for 2 minutes and the supernatant was transferred to a new tube. This supernatant contained the cytosolic fraction. To the pellet, 50 µl of ice-cold buffer C (10 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 5 µg/ml PIM) was added, incubated in ice for 15 minutes and then centrifuged at 13,000 rpm for 15 minutes. The supernatant having the nuclear extract was transferred to a fresh tube and both the fractions were kept frozen at –80^{o}C until further use.

2.21 Protein estimation

The protein content in extracts was estimated using the Pierce coomassie protein assay reagent (Pierce Biotechnology, Rockford, USA). This method is based on the fact that when coomassie dye binds protein in an acidic medium, an immediate shift in absorption maximum occurs from 465 nm to 595 nm with a concomitant color change from brown to blue. Lysates where diluted with water in a 1:50 to 1:200 ratio. BSA in the range 0 - 25 µg/µl was used for the standard curve. The Pierce reagent was added in a ratio of 1:1 in a microtitre plate and the blue colour developed was measured spectrophotometrically by means of a microplate reader (Dynex Technologies, Berlin, Germany).

2.22 Western Blot Analysis

The protein samples were prepared and separated by SDS-PAGE as mentioned earlier. Proteins were transferred to a nitrocellulose membrane wetted with the transfer buffer (methanol 20% (v/v), Tris base 25 mM, glycine 192 mM) using a semi-dry electroblotter. It was used in accordance with the manufacturer's instructions with protein transfer performed at 100 V for 1 hour. Protein transfer was assessed by the complete transfer of prestained low molecular weight

markers on the membrane. The membrane was then blocked for at least two hours at room temperature in blocking buffer - TBST (25 mM Tris-HCl, pH 8.0, 125 mm NaCl, 0.1% Tween 20) with 5% milk powder. This was then decanted and replaced with the primary antibody solution diluted appropriately in the blocking buffer. The membrane was incubated in this mixture overnight at 4^0C with gentle agitation. The membrane was then washed 5 times for 5 minutes each in TBST after which a horse-radish peroxidase conjugate was added to it appropriately diluted in the blocking buffer. After an incubation of 1 hour at room temperature with gentle agitation, the membrane was washed with TBST 5x for 5 minutes. Bound antibodies were then detected on the membrane using ECL reagent in accordance with the manufacturer's instructions.

2.23 Immunoprecipitation

COS-1 cells were transfected in 10 cm dishes with the plasmid for the over expression of the protein/s of interest, lysed in 1 ml ice-cold lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 0.1 mM EGTA, 1 mM Na_3VO4, 0.5% deoxycholic acid, 0.1% SDS, 10 mg/ml aprotinin, 10 mg/ml pepstatin, and 20 mM phenylmethylsulfonyl fluoride) and sonicated (4 output control, 20% duty cycle, 3 x 5 seconds each) on ice. Cell debris were removed by centrifuging at 17000 rpm for 20 minutes at 4^0C. The supernatant was transferred to a fresh eppendorf tube and 50 μl from this was kept aside to check for the protein expression by SDS-PAGE and western blot analysis. The rest of the supernatant was divided into two eppendorf tubes and 2 - 5 μl of the pre-immune sera or the specific antibody was added to each separately. The tubes were incubated at 4^0C for 1 hour on an end-to-end shaker. 5 μl of Sepharose beads were then added to both and incubated again for 1 hour in the same conditions. The beads were collected by a brief centrifugation step and washed 3 times with lysis buffer. At the end of the last wash, the entire buffer was aspirated from the beads, 20 -30 μl of loading buffer was added and the proteins analysed by SDS-PAGE and western bloting.

2.24 Expression and purification of GST-fusion proteins

The plasmid (pGEX) coding for the protein expressed as a GST-fusion was transformed in *E.coli* BL21. A single colony was inoculated in 5 ml of LB media with the appropriate selection marker and incubated overnight at 37^0C at 250 rpm. Fresh LB media was inoculated with the overnight culture in the ratio of 1:25 and incubated at 37^0C at 250 rpm. After 1 hour, the culture was induced with IPTG at a final concentration of 0.1 mM and incubated at 30^0C - 36^0C at 250 rpm for 2 - 4 hours. The cells were harvested and the GST fusion protein was bound to glutathione sepharose 4B beads according to the manufacturer's protocol.

2.25 GST-pulldown

COS-1 cells were transfected in 10 cm dishes with the plasmid for the over-expression of the protein of interest, lysed in 1 ml ice-cold lysis buffer (cf preceding passage) and sonicated on ice as described above. Cell debris was removed , the supernatant was transferred to a fresh eppendorf tube and 50 μl from this was kept aside to check for the protein expression by SDS-PAGE and western blot analysis. To 500 μl of the supernatant was added 5 μl of the glutathione sepharose 4B beads loaded with the GST fusion protein. The tube was incubated at 4^0C for 1 hour on an end-to-end shaker. The beads were collected by a brief centrifugation step and washed 3 times with ice cold PBS. At the end of the last wash, the entire buffer was aspirated from the beads, 20 - 30 μl of loading buffer was added and the proteins analysed by SDS-PAGE and western blotting.

2.26 Yeast interaction trap assay

Yeast interaction trap assay vectors pLexA and pJG4-5 and yeast strains EGY48 (MATα trp1 his3 ura3 leu2::6LexAop-LEU2 containing the reporter plasmid pSH18-34) and RFY206 (MAT a trp1::hisG his3 δ200 ura3-52 lys2 δ201 leu2-3)

for the interaction trap assay were generously provided by Roger Brent (Boston). For mating, cell suspensions of both strains (50 µl each) were mixed and incubated in rich medium (YPD) at 30^0C for 14 - 16 h. Interactions were validated by growth and blue coloring on minimal agar plates lacking uracil, histidine, tryptophan, and leucine, supplemented with 2 % galactose, 1 % raffinose, and 80 µg/ml 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal).

2.27 Spermidine/spermine acetyltransferase activity

20 µL of sample containing the desired concentration of SSAT-GST was put into chilled 1.5 mL eppendorf tube in duplicate. 30 µL 0.5M HEPES was used for the blank (lower limit of activity). 30 µL of reaction mixture (10 µL 0.5 M HEPES, pH 7.8, 5 µL 30 mM spermidine, 0.5 µL [^{14}C]acetyl coenzyme A- (Amersham Biosciences, UK, in 14.5 µL dd H_2O) was then added to each tube except the blank. 30 µL of the reaction mix was aliquoted into two scintillation vials for the TSC (total substrate count- upper limit of activity). Tubes were vortexed and placed on rack of tubes uncapped in 37°C water bath for 5 minutes. The rack was placed back on ice and 20 µL of 0.5 M hydroxylamine was added to each tube. Samples were boiled for 3 minutes. Whatman p81 filters (Markson Lab Sales, USA) were labeled with pencil for each sample and blank and placed on non-absorbent surface material. 50 mL of each sample was placed onto each filter. The sample-soaked filters were placed into a wash system with a continuous slow-flow of distilled water for 30 minutes. Filters were removed and dried on Whatman #1 filter paper. Each filter/sample was placed into a scintillation vial, labeled, suspended in 5 mL non-aqueous counting fluid (Biosafe, USA) and each sample was counted for 1 minute.

III Results

1 Transcriptional regulation of sGC expression

The 5' upstream region of the rat α−1 and the β−1 sGC genes had been isolated by rapid amplification of cDNA ends (RACE) and analyzed for promoter activity by using luciferase reporter constructs. Their transcriptional start sites had been identified by Dr. Meik Behrens in our laboratory in primary smooth muscle cells. It was clear that both genes are transcribed independently; however the regulation of each promoter remained to be elucidated. In order to determine the promoter region harboring the maximal basal activity, deletion constructs of the sGC α−1 promoter were analysed for the reporter activity. The pivotal transcription factors responsible for the activity of this fragment were identified. However, to establish the transcriptional regulation of the gene, it was important to analyse the promoter response to an external stimulus and to investigate the contribution of the identified transcription factors. Various experiments were designed for this study the results of which are described in the following chapters.

1.1 Basal activity of the promoter of sGC α-1 subunit in RLF-6 cells

Genome walking from the 5' UTR of the α−1 subunit resulted in different clones that were subsequently sequenced and cloned into the pGL3basic vector containing luciferase as reporter gene. For the α−1 subunit following clones were obtained Alpha3000 (with -2794 bp), Alpha1100 (-1092 bp), Alpha350 (-346 bp) and Alpha200 (-200 bp) (see fig. 8).

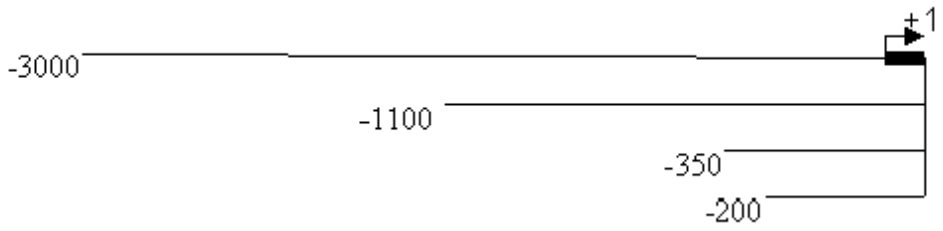


Figure 8: The 5' UTR of the rat sGC α–1 gene and deletional constructs. The deletion constructs were cloned into pGL3 vectors. Position of the identified transcriptional start site is indicated as +1.

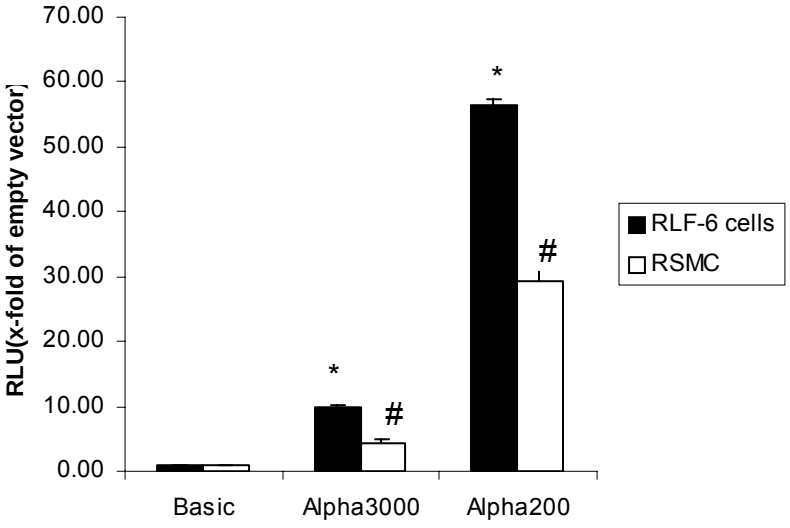


Figure 9: Promoter activity of sGC α-1 deletion constructs in cultured rat aortic smooth muscle cells and RLF-6 cells. The constructs were transfected in the cells transiently and the reporter activities were determined. The firefly luciferase activity was normalised by the renilla luciferase activity. Transfections were performed in triplicate. Results are means ± SD (n=3) *, # P<0.05; ANOVA.

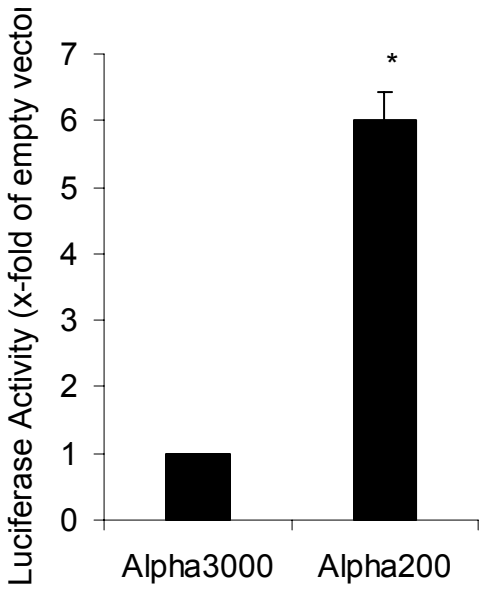


Figure 10: Promoter activity of sGC α–1 deletion constructs in RLF-6 cells. RLF-6 cells were transfected with luciferase reporter constructs containing different size promoter fragments and reporter activities were determined. The luciferase activity produced by the pGL3-basic or pGL3-Alpha-sGC was normalised by the luciferase activity produced by pRL-TK and are expressed in relative light units. Transfections were performed in triplicate. Results are means ± SD (n=3) * P<0.05.

The activity of the various deletion constructs of the α–1 sGC promoter was established in RLF-6 cells using luciferase reporter constructs and was found to be the same as in cultured rat aortic smooth muscle cells as observed by Dr. Meik Behrens in his doctoral thesis. However, the activity of the constructs were much lower (about five fold low) in smooth muscle cells as compared to the RLF-6 cell line. This cell line consistently expresses high levels of the α–1/β–1 sGC heterodimer. Thus the rat lung fibroblast RLF-6 cell line was chosen as a cell model to investigate transcriptional activity in the subsequent experiments. Transiently transfected luciferase reporter constructs harboring 0.2-3.0 kb of α–1 sGC upstream sequence were analysed for promoter activity. The Alpha200 construct containing ~200bp upstream the transcription start showed highest level of activity (fig. 9, 10).

2 Identification of transcription factors responsible for the basal expression of α-1 subunit of sGC in RLF-6 cells

We found that the short core promoter of 200 bp upstream the transcription start site exhibited the highest basal promotor activity in reporter assays. We hypothesized that this core promotor decisively controls constitutive expression of the sGC α−1 subunit. Therefore, we were interested to identify the transcription factors responsible for this basal activity, in order to provide a mechanistic basis for delineating potential signalling pathways, which could regulate transcription of sGC subunit. Therefore, the following experiments were designed to study the effects of inhibiting the binding of these on the short as well as the full-length promoter. Analysis of the 200 bp of the 5' UTR of the α−1 gene was performed using the MATINSPECTOR V2.2 software and those transcription factors were included in the study which had a core similarity of 1:00 and matrix similarity of 0.95. The contribution of these transcription factors towards the basal regulation of the α−1 subunit of sGC was investigated by the following methods.

2.1 Decoy oligonucleotide approach

To investigate the various transcription factors binding to the promoter of sGC α−1, RLF-6 cells were transiently co-transfected with the Alpha200 construct and 10 nM of the decoy oligonucleotides (ODN) for NFY (CCAAT binding protein), Sp1, CABL (C-abl binding protein) and MZF (myeloid zinc finger protein). These factors were used because these were found by in silico analysis as described in above. The sequences of the single-stranded ODN were as follows (Table 6):

Table 6: Decoy oligonucleotides for NFY, Sp1, CABL and MZF (-pto: the first and the last 4 bases are phosphorothioated for use as decoy)

cablf pto - 5'-aaaaGGTTTTTGTTTTtaac-3' bp 2686 – bp 2715

cablr-pto - 5'-gttaAAAACAAAAACCtttt-3'

sp1f-pto - 5'-gcgaTTGGGCGGGGAGcagg-3' bp 2808 – bp 2827

sp1r-pto - 5'-cctgCTCCCCGCCCAAtcgc-3'

nfyf-pto - 5'-ggacAGCGATTGGGCGggga-3' bp 2803 – bp 2822

nfyr-pto - 5'-tcccCGCCCAATCGCTgtcc-3'

mzf1f-pto - 5'-attgGGCGGGGAgcag-3' bp 2811 – bp 2827

mzf1r-pto - 5'-ctgcTCCCCGCCcaat-3'

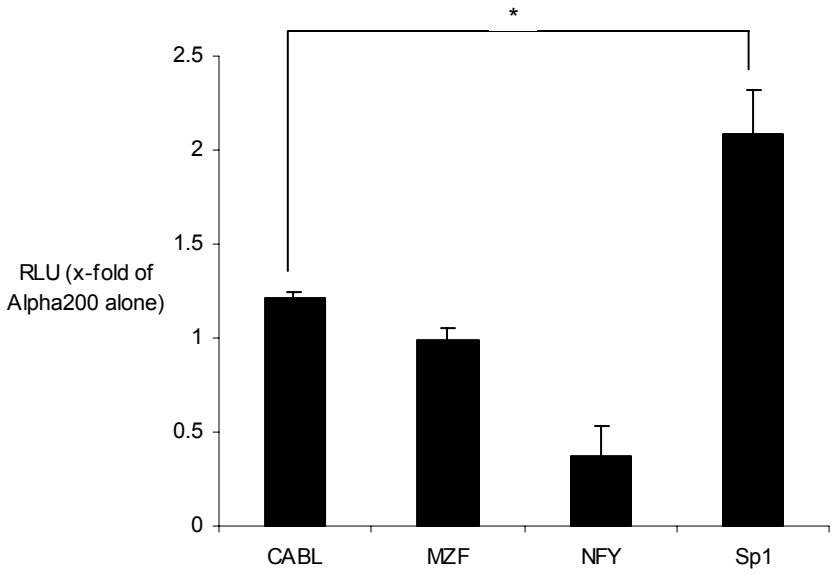


Figure 11: Effects of the decoy oligodeoxynucleotides for NFY, Sp1, CABL and MZF on reporter gene expression of the Alpha200 construct in RLF-6 cells. 10 nM of the oligos were co-transfected with the Alpha200 construct in RLF-6 cells. After a 24-hour incubation period in the absence of serum, the luciferase activity was measured and normalised to the renilla counts. Transfections were performed in duplicate. Results are means ± SD (n=3) *P<0.05; ANOVA.

The promoter activity (reporter gene expression) of the Alpha200 construct was almost doubled by treatment of the cells with the Sp1 ODN, whereas treatment with the NFY ODN resulted in a significant decrease in promotor activity (fig. 11). These findings suggest that the transcription factors Sp1 and NFY play an important role in the regulation of basal sGC α-1 transcription. In contrast, Alpha200 promotor activity was not significantly affected by exposure of the cells to the MZF and CABL ODNs (fig. 11).

2.2 Effect of core deletions on basal promoter activity

To confirm that the NFY and Sp1 binding sites are crucial for α–1 sGC promoter activity, we introduced small deletions in the Alpha200 construct containing the 200 bp fragment. We chose this fragment because it possessed maximal promoter activity. In addition, we determined the effect of these deletions on the promoter activity of the Alpha3000 construct, which would reveal the influence of the other parts of the promoter on core promotor activity. Putative binding sites for CAAT and Sp1 transcription factors were investigated. The cores of these transcriptional factor binding sites were deleted individually in the constructs via site-directed mutagenesis. Boxes in fig. 12 denote the deleted nucleotides. The core deletions did not introduce any new binding sites as confirmed by sequence analysis with MATINSPECTOR V2.2 software. Transcriptional activities of the generated mutant constructs were assessed in RLF-6 cells.

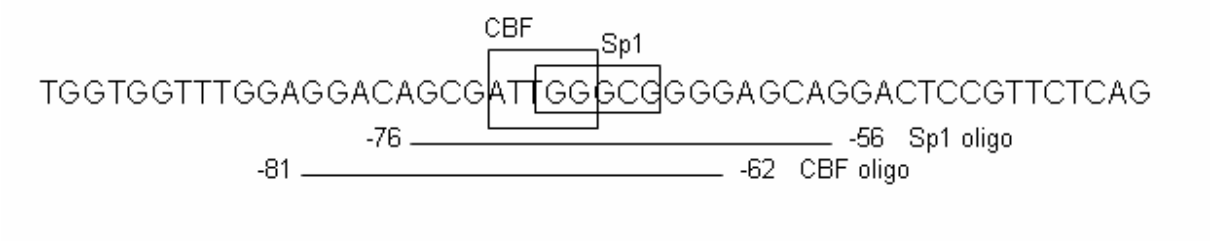


Figure 12: The proximal promoter sequence of the rat α–1 sGC gene. Letters in boxes indicate the core binding sites for NFY and Sp1. The oligo-deoxynucleotides for EMSA for these factors are underlined.

sp1_for - 5'-GGAGGACAGCGATT***gggcgg***GGAGCAGGACTCCGTTCTC-3' bp 2800 – bp 2838

sp1_rev - 5'-GAGAACGGAGTCCTGCTCCAATCGCTGTCCTCC-3'

nfy_for - 5'-GGTTTGGAGGACAGCG***attgg***GCGGGGAGCAGGACTCCG-3' bp 2795 – bp 2833

nfy_rev - 5'-CGGAGTCCTGCTCCCCGCCGCTGTCCTCCAAACC-3'

nfy2f - 5'-GATTGACAAGCCATTAG***ccaat***CTGTTGAGAATGAGGAAG-3' bp 618 – bp 657

nfy2r - 5'-CTTCCTCATTCTCAACAGCTAATGGCTTGTCAATC-3'

gcf - 5'-CCTGACATGCACGCCACC***cccccc***CACCACACACACCCACAC-3' bp 1270 – bp 1311

gcr - 5'-GTGTGGGTGTGTGTGGTGGGTGGCGTGCATGTCAGG-3'

Figure 13: ODN for site-directed mutagenesis. Forward and reverse primers for each transcription factor are shown. Letters in bold indicate the cores of binding sites for transcriptional factors NFY and Sp1 that were deleted in the reporter constructs.

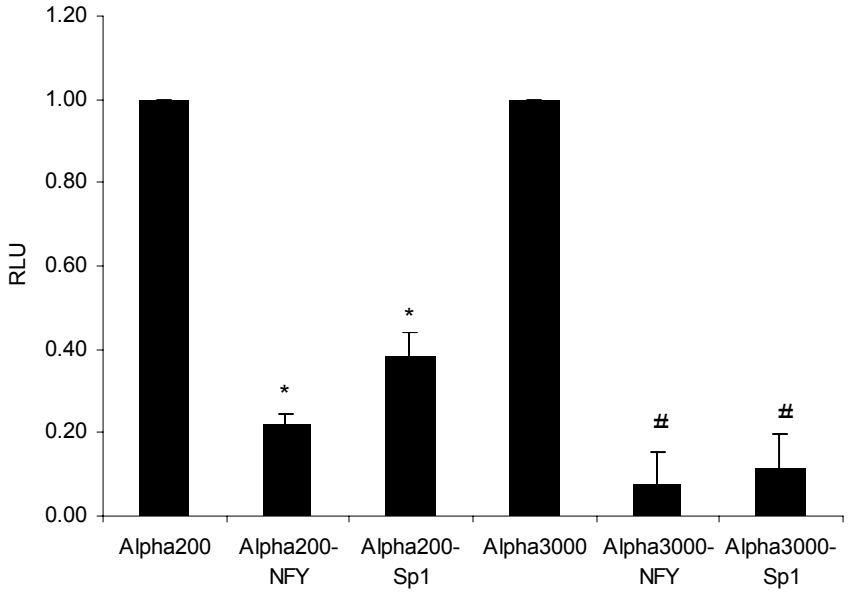


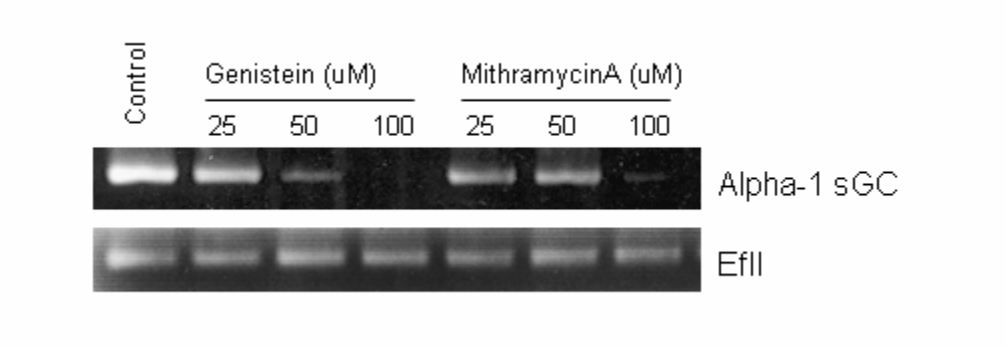
Figure 14: Effect of core deletions for NF-Y and Sp1 on the activity of Alpha200 and Alpha3000 constructs in RLF-6 cells. RLF-6 cells were transfected with the constructs either with or without the deletion, which were the controls. After incubation for 16-20 hours in the absence of serum, cell lysates were assayed for luciferase activity and compared (Controls=1 RLU. Transfections were performed in triplicate. Results are in mean ± SD (n=3) *, # $P<0.05$; ANOVA.

The site directed mutations using the ODNs mentioned in fig. 13 resulted in the constructs Alpha200-NFY, Alpha200-Sp1, Alpha3000-NFY and Alpha3000Sp1. These constructs were used for transfecting RLF-6 cells after which they were incubated for a further 16-20 hours in the absence of serum. As seen in fig. 14, the constructs containing the deleted sites for NF-Y and Sp1 showed a significant decrease in constitutive promotor activity, implying that these transcription factors are crucial elements in the basal expression of the of sGC α–1 subunit.

2.3 Decreased expression of sGC α–1 by inhibitors of DNA binding

To investigate the effect of inhibiting the interaction of NFY and Sp1 transcription factors with the promoter on sGC α–1 mRNA expression, RLF-6 cells were treated with varying concentrations of genistein and mithramycin A for 16 hours. Total RNA was extracted and reverse transcription and polymerase chain reaction were performed. Genistein inhibits the binding of NFY to the CCAAT box possibly by phosphorylation of the NFY-B subunit (Zhou Y, 1998). Mithramycin binds to GC boxes and prevents sequential binding of Sp1 (Blume SW, 1991). Both genistein and mithramycinA induced a concentration-dependent decrease in the expression of the sGC α–1. Genistein 50 µM and mithramycinA 100 nM were chosen as the optimum concentration required to see a significant effect and were used for subsequent experiments (fig. 15). This finding also implied that both NFY and Sp1 have an activating effect in the full-length sGC α–1 promoter.

A)



B)

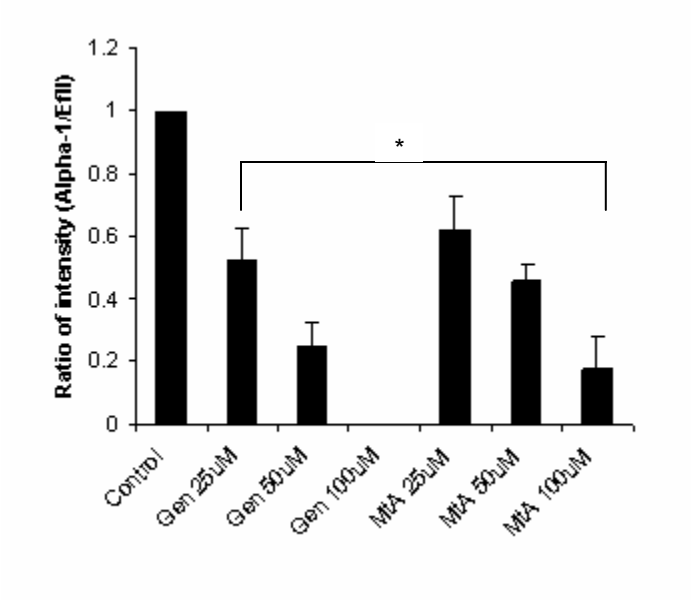


Figure 15: RT-PCR . RLF-6 cells serum-starved for 12-16 hours were incubated with increasing doses of mithramycin A (MtA) and genistein (Gen) for 16-20 hours. RNA was prepared and RT-PCR was carried out for sGC α–1. **A)** The PCR products were electrophoresed on a 1 % agarose gel and stained with ethidium bromide. **B)** Densitometric analysis was performed on the gel and the intensities of the fluorescent bands were determined and plotted on the graph. * P<0.05; ANOVA n=3.

2.4 Decreased promoter activity by inhibitors of DNA binding

To investigate the importance of the interaction of NFY and Sp1 transcription factors with the Alpha200 promoter on its activity, RLF-6 cells were transiently co-transfected with Alpha200 construct and treated with genistein and mithramycinA in the absence of serum. A 16-hour treatment with 50 µM genistein inhibited the activity by 50 % whereas treatment with 100 nM mithramycinA, increased the activity by almost two-fold (fig. 16).

To confirm that the effect of the inhibitors on the sGC α–1 expression is transcriptional, RLF-6 cells were transfected with the longest promoter construct Alpha3000, and similarly treated with 50 µM genistein and 100 nM mithramycinA for 16 hours before harvesting. Both the agents induced a significant decrease of the promoter activity by 50 % (fig. 17).

The opposite effects of mithramycinA in the Alpha200 andAlpha3000 constructs indicates that there is another Sp1 binding site present in the full-length promoter construct which overcomes the activation of the Sp1 site in the Alpha200 region in response to mithramycinA. Thus we see an overall reduction in mRNA expression and full-length promoter activity as well.

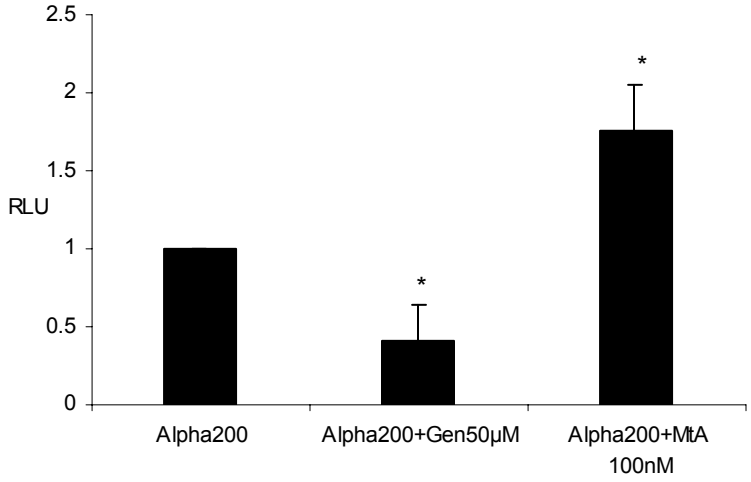


Figure 16: Effect of the inhibitors of NFY and Sp1 (genistein and mithramycin) on the activity of the Alpha200 construct in RLF-6 cells. RLF-6 cells were transfected with the Alpha200 construct and treated with 50 µM genistein and 100 nM mithramycin A for 16 hours in the absence of serum. Cell lysates were assayed for luciferase activity and compared with untreated controls. Transfections were performed in triplicate. Results are in mean ± SD (n=3) * P<0.05; ANOVA.

To confirm that the effect of the inhibitors on the sGC α–1 expression is transcriptional, RLF-6 cells were transfected with the longest promoter construct Alpha3000, and similarly treated with 50 µM genistein and 100 nM mithramycin A for 16 hours before harvesting. Both the agents induced a significant decrease of the promoter activity by 50 % (Fig. 23).

The opposite effects of mithramycin A in the Alpha200 and Alpha3000 constructs indicates that there is another Sp1 binding site present in the full-length promoter construct which overcomes the activation of the Sp1 site in the Alpha200 region in response to mithramycin A. Thus we see an overall reduction in mRNA expression and full-length promoter activity as well.

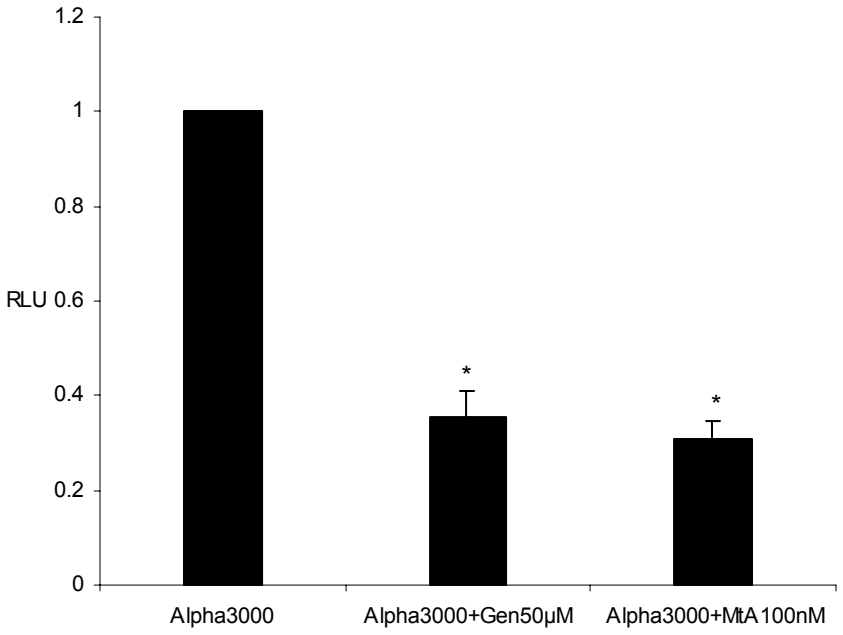


Figure 17: Effect of genistein and mithramycinA on the activity of Alpha3000 in RLF-6 cells. RLF-6 cells were transfected with Alpha3000 and treated with 50 µM genistein and 100 nM mithramycinA for 16 hours in the absence of serum. Cell lysates were prepared and the luciferase activity was measured and normalised to the renilla luminescence counts. Transfection was performed in triplicate. Results are in mean ± SD (n=3) * P<0.05; ANOVA.

2.5 DNA binding sites for the transcription factors NFY/Sp1

In order to identify proteins bound to the potential transcription factor binding sites found in the analysis of the sGC α–1 promoter, nuclear extracts from RLF-6 cells serum-starved for 16-20 hours were analysed by EMSA using biotin labelled DNA sequences of binding sites for the respective transcription factors. The probes used contained the following regions upstream of the transcriptional start site for the NFY and Sp1 ODNs respectively and were biotinylated at the 5' end:

Table 7: **Biotin labelled probes used for EMSA for the Sp1 and NFY transcription factors.**

sp1f-bio - 5'BIO-gcgattgggcggggagcagg-3' bp 2808 – bp 2827

sp1r-bio - 5'BIO-cctgctccccgcccaatcgc-3'

nfyf-bio - 5'BIO-ggacagcgattgggcgggga-3' bp 2803 – bp 2822

nfyr-bio - 5'BIO-tccccgcccaatcgctgtcc-3'

The EMSA clearly showed the formation of multiple complexes with the biotinylated ODN probes for NFY and Sp1 when incubated with RLF-6 nuclear extract (fig. 18), as can be seen in lanes 1. In order to reveal the presence of these transcription factors in the shifted bands an antibody directed against either Sp1 or NFY protein was co-incubated with the nuclear extract and the respective oligodeoxynucleotide probe. In the presence of the antibody, a new shifted band appeared, while the intensity of one of the shifted bands seen in the absence of antibody decreased. This so-called "supershift" was induced by interaction of the antibody with the transcriptionfactor-oligo-DNA complex. A gradually increasing supershift was observed on incubating the complexes with increased concentrations of the antibodies for NF-Y and Sp1 (lanes 2 and 3). This finding demonstrates that these transcription factors were present in the shifted band, indeed. This experiment provides evidence that both transcription factors are present in nuclear extract from RLF fibroblasts and can bind to the core region of the sGC α–1 promoter.

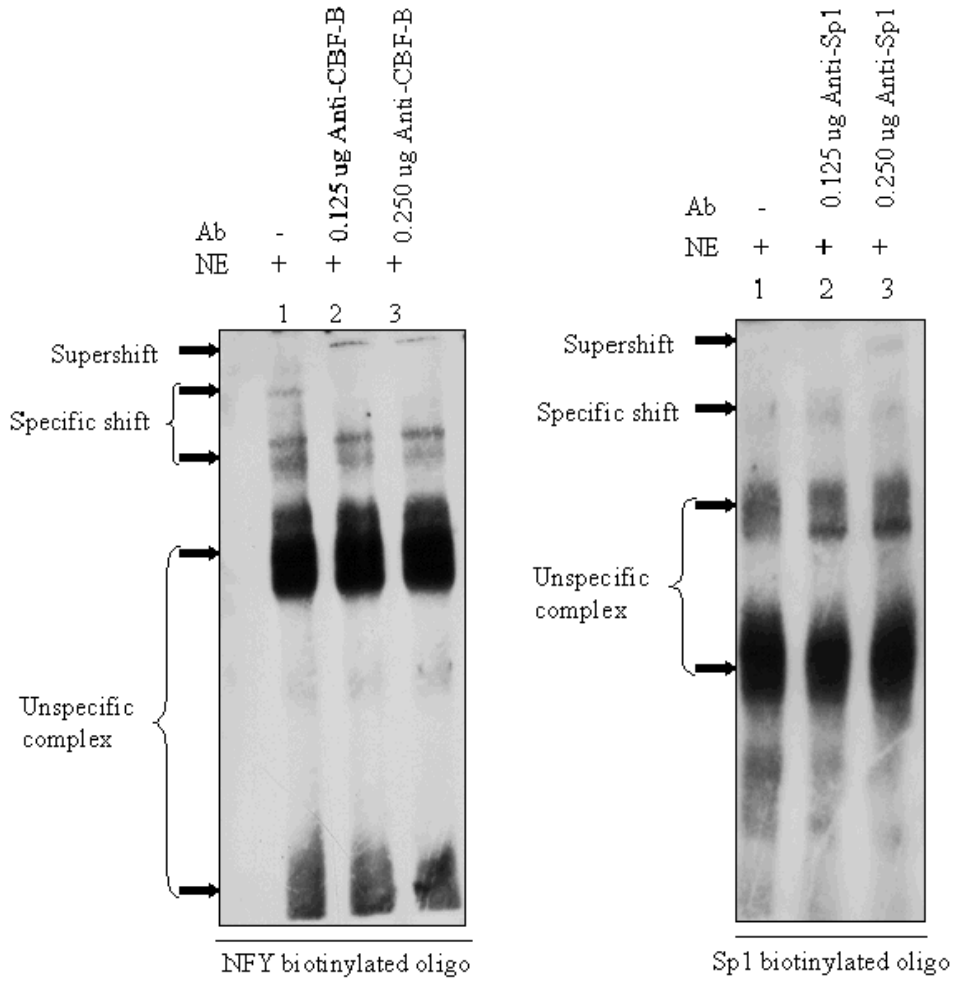


Figure 18: EMSA performed with 1 μg nuclear extract (NE) from RLF-6 cells incubated with 2 ng of the biotinylated NF-Y and Sp1 oligodeoxynucleotides. A supershift (indicated by arrow) was performed by adding increasing concentrations of the respective antibodies. The complexes were separated on a polyacrylamide gel and transferred onto nylon membranes. The detection of the biotin labelled DNA was carried out using the LightShift chemiluminiscent EMSA kit. Representative EMSA from three experiments are shown.

A gradually increasing supershift was observed on incubating the complexes with increased concentrations of the antibodies for NF-Y and Sp1 (lanes 2 and 3). This finding demonstrates that these transcription factors were present in the shifted band, indeed. This experiment provides evidence that both transcription factors are present in nuclear extract from RFL fibroblasts and can bind to the core region of the sGC α–1 promoter.

2.6 Specificity of the decoy approach for inhibition of sGC α–1 promoter activity

To examine the specificity of the consensus decoy ODN for NFY and Sp1, an electrophoretic mobility shift assay (EMSA) was performed with nuclear extract from RLF-6 cells transfected with 10 nM of these decoy ODN for 12 hours in the absence of serum and the respective biotinylated oligodeoxynucleotides. The bindings of NFY and Sp1 oligos were significantly reduced in the nuclear extracts from NFY and Sp1 decoy ODN treated cells respectively (fig. 19). This indicates that the abundance of the transcription factor was significantly reduced in nuclear extracts of cells transfected with the respective decoy ODN. Also, the decoy ODN treatment specifically reduced the binding capacity of the targeted transcription factor, not that of other transcription factors, i.e. the Sp1 binding, for instance, was not affected in NFY decoy ODN transfected cells and vice-versa.

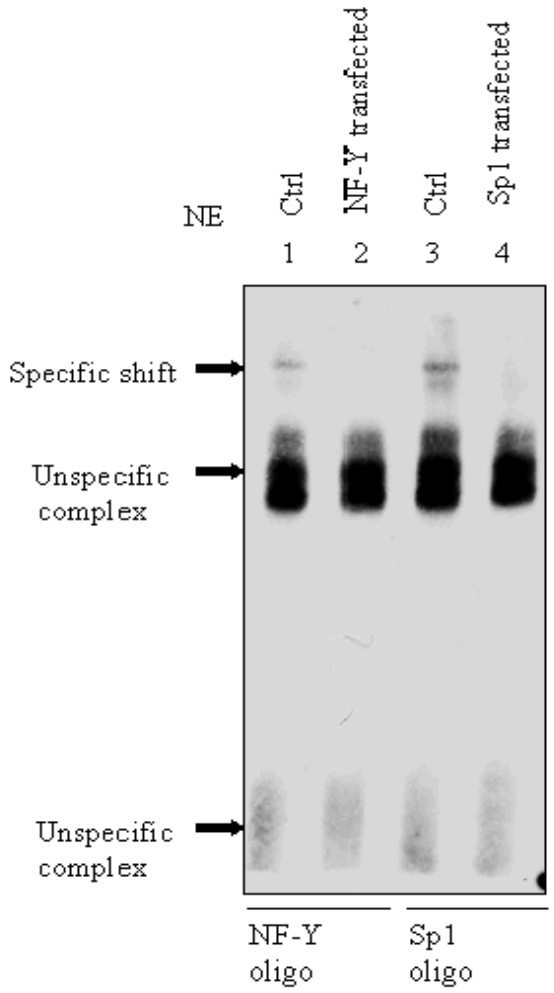


Figure 19: Specificity of the decoy oligodeoxynucleotides. RLF-6 cells were transfected with 10 nM of the NFY and Sp1 decoy ODN and incubated for 12 hours in the absence of serum. Nuclear extract was prepared and EMSA was performed with 1 μg of the extract and 2 ng of the respective biotinylated oligos. Untransfected cell lysate was used as the controls. Representative EMSA out of 3 experiments is shown.

2.7 Competitive inhibition of transcription factor binding

To demonstrate the specificity of the complexes formed, increasing concentrations of unlabeled NF-Y/Sp1 consensus oligos were added to the cell lysate and biotinylated NFY/Sp1 oligo. A reduction of the band intensities in the presence of a 5-fold excess of unlabeled consensus probe (fig. 20) was observed. This indicates that there was competition of binding of the transcription

factors for labelled and unlabelled consensus DNA probes and with increased concentration of unlabeled oligo, lesser of the factors were binding to the biotinylated oligo; hence the reduction in intensity (fig. 20, lanes 2 and 3; lanes 5 and 6).

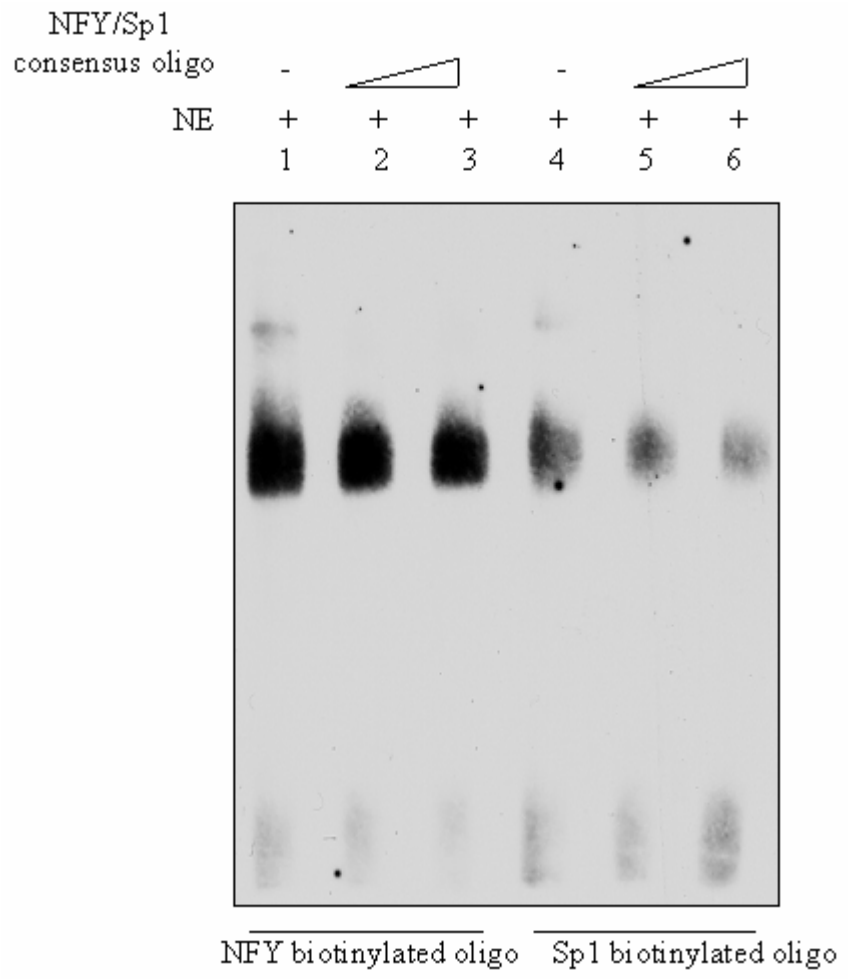


Figure 20: Competitive EMSA. 0.5 μg nuclear extract from RLF-6 cells serum-starved for 16-20 hours were incubated with 2 ng of the biotinylated NF-Y and Sp1 oligos. Competiton for binding was performed by adding increasing concentrations of the respective unlabeled consensus oligos. The complex were separated on a polyacrylamide gel and transferred onto nylon membranes. The detection of the biotin labelled DNA was carried out using the LightShift chemiluminiscent EMSA kit. Representative of three independent experiments are shown here.

With increased concentration of unlabeled oligo, lesser of the factors were binding to the biotinylated oligo; hence the reduction in intensity of bandshift was seen (Fig. 20, lanes 2 and 3; lanes 5 and 6).

2.8 Reduction in enzyme activity by the inhibitors of DNA binding

In order to check whether the decrease in the transcription level induced by inhibition of binding of these transcription factors to the promoter resulted also in a decrease in the protein level of the sGC α–1 subunit and hence a decreased enzyme activity, RLF-6 cells were treated with genistein 50 µM and mithramycin A 100 nM for 16 hours. Thereafter, the cells were washed with PBS and isobutylmethylxanthine (IBMX, 0.5 mM) was added in order to prevent degradation of cGMP by cGMP-specific phosphodiesterases. 10 minutes later 100 µM sodium nitroprusside (SNP) was added in order to maximally activate NO-sensitive sGC in these cells. After further 5 minutes the PBS was removed and the cells were denatured and lysed by adding 0.1 N HCl (0.5 ml per well). The cell lysates were evaluated for their cGMP content by enzyme-linked immunoassay (EIA, Amersham Biosciences). When stimulating maximally by NO, this is an estimate for the amount of functionally intact enzyme present in the cells. The activity of the treated cells were compared with the solvent-treated controls. The amount of cGMP detected (fmol/well) was normalized by the cellular protein content of the samples. As shown below the cGMP content of the cells was significantly reduced by both inhibitors (fig. 21), almost completely by genistein, and by about 40 % by mithramycin. This decrease in cGMP was not due to an unspecific toxic effect exerted on the cells, as verified by trypan blue staining and microscopic inspection. This experiment showed that chemicals interfering with the constitutive activity of the transcription factors NFY and Sp1 reduced the content of functionally competent sGC, and furthermore showed that the level of the functional holoenzyme depends on the mRNA expression of the of the sGC α-1 subunit.

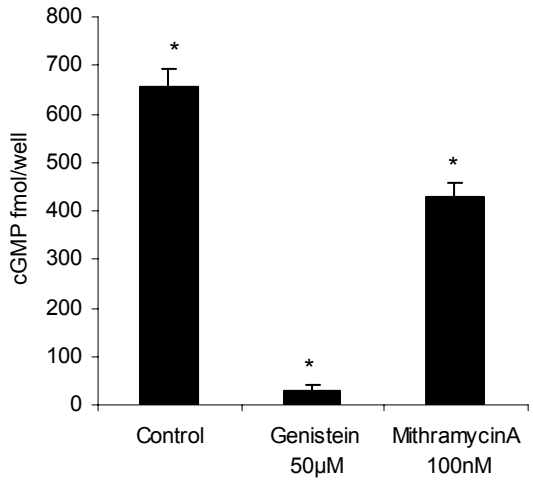


Figure 21: Effect of Genistein and MithramycinA on the cGMP content in RLF-6 cells. RLF-6 cells were treated with 50 µM genistein and 100 nM mithramycin A for 16 hours. The cGMP content was measured using Enzyme Linked Immunoassay. Incubations were performed in triplicate. The results are expressed as mean ± SD (n=3) * P<0.05; ANOVA.

3 Effect of serum on sGC α-1 expression regulation

NFY and Sp1 are instrumental in several physiological and pathophysiological effects mediated by several growth factors in smooth muscle cells. Sp1 plays an important role in the TGFβ mediated induction of integrins, which in turn is important in restenosis and atherosclerosis (Kintscher U, 2002). It mediates PDGF-induced p21 gene expression in VSMC which been shown to be a component of active cyclin/CDK complexes (Moon SK, 2003). Sp1 is also involved in the suppression of SMC differentiation marker gene expression in experimental atherosclerosis by PDGF (Wamhoff BR, 2004). NFY mediates PDGF induced Notch-3 receptor expression and signaling in vascular smooth muscle cells which has implications in cell-cycle regulation (Campos AH, 2002). Serum is considered to be a good source of many growth factors as TGF, PDGF, FGF, VEGF and EGF (Andreeva V, 2004);(Brown MR, 1991);(Chen SC, 1982). It would be possible that at least one of these NFY or Sp1 mediated processes by any one of the growth factors actually occurs involving the regulation of

expression of sGC. Thus we decided to use serum as a stimulus to study the response of the sGC α−1 promoter.

3.1 Serum downregulates sGC α−1 protein expression in rat aorta

The effect of FCS on sGC α−1 protein expression was investigated by incubating isolated rat aortic segments (10 mm length) for 24 hours in culture medium supplemented with 10% FCS.

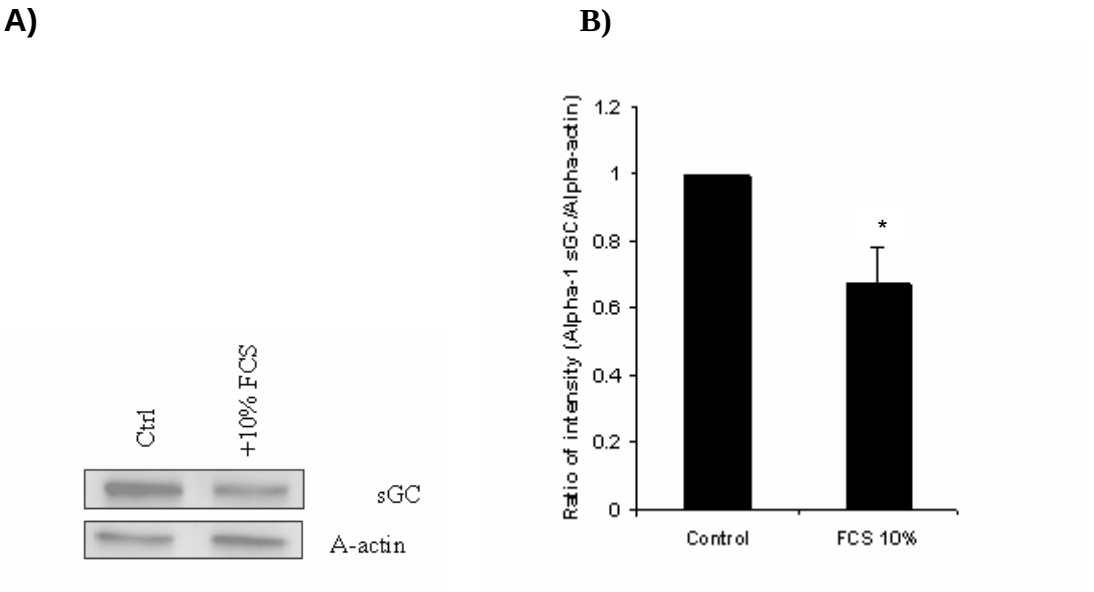
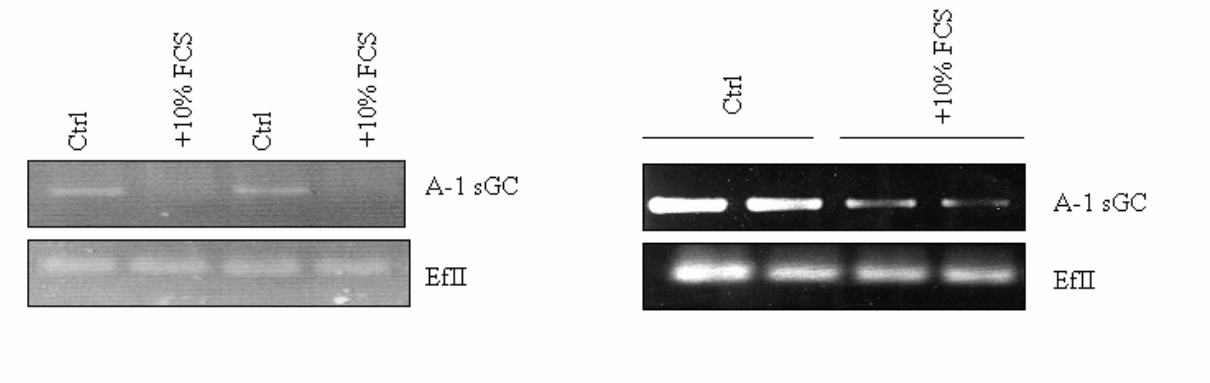


Figure 22: Effect of FCS on expression of sGC α−1 protein in rat aorta. Isolated segments of rat aorta were incubated for 24 hours with cell culture medium supplemented without (Control) or with 10% FCS. Cell lysate was prepared and a western blot was carried out for sGC α−1. **A)** Anti-α−1/β−1-sGC antibody was used as the primary antibody (1:400) and anti-chicken antibody as the secondary antibody (1:5000). **B)** Densitometric analysis was performed on the gel and the intensities of the bands were determined and plotted on the graph. Mean values ± SD from n = 3 * P<0.05.

The effect of FCS on sGC α–1 protein expression was investigated by incubating isolated rat aortic segments (10 mm length) for 24 hours in culture medium supplemented with 10% FCS. Thereafter the aortic tissue was ground in liquid nitrogen by means of a pestle and mortar, the protein was extracted from the tissue powder by vortexing with hypotonic buffer solution (see Methods), and a western blot was performed for analysing sGC α–1 protein expression (fig. 22). The blot clearly showed a decreased expression (by one third) upon incubation with FCS as compared to organ incubation in its absence, indicating that FCS down regulated sGC α–1 expression.

3.2 Serum downregulates sGC α-1 mRNA expression in RLF-6 and RASM cells

A)



B)

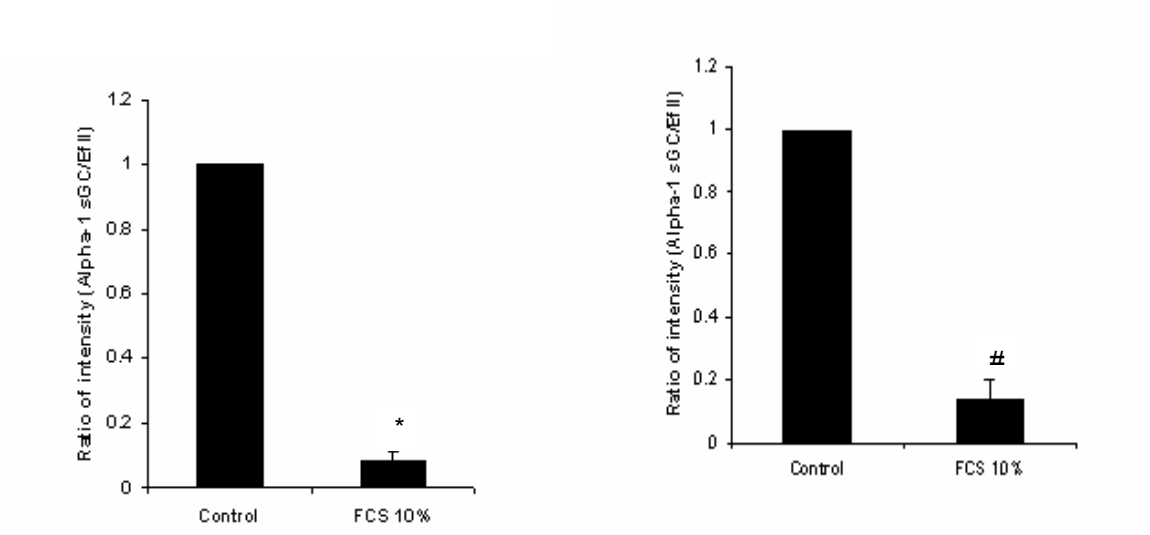


Figure 23: Effect of FCS on sGC α–1 mRNA expression in cultured rat aortic smooth muscle cells and RFL-6 cells. Cells were incubated for 24 hours in the absence (Control) and presence of 10% FCS. RNA was prepared (see Methods) and RT-PCR was carried out for sGC α–1. A) The PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. B) Densitometric analysis was performed on the gel and the intensities of the bands were determined and plotted on the graph. Mean values ± SD from n = 3 *, # P<0.05; ANOVA.

In order to assess whether FCS decreases the expression of sGC α–1 also at the mRNA level, cultured rat aortic smooth muscle cells and RLF-6 cells were grown for 24 hours in the absence and presence of 10% FCS, the mRNA was extracted from the cell lysates, an RT-PCR for sGC α–1 mRNA was performed (see Methods) As illustrated in fig. 23 there was a clear reduction of mRNA expression in both the cell types following FCS incubation. This finding further lends support to our theory that growth factors present in FCS reduce the expression of sGC α–1.

3.3 Decrease in sGC α-1 promoter activity in response to serum

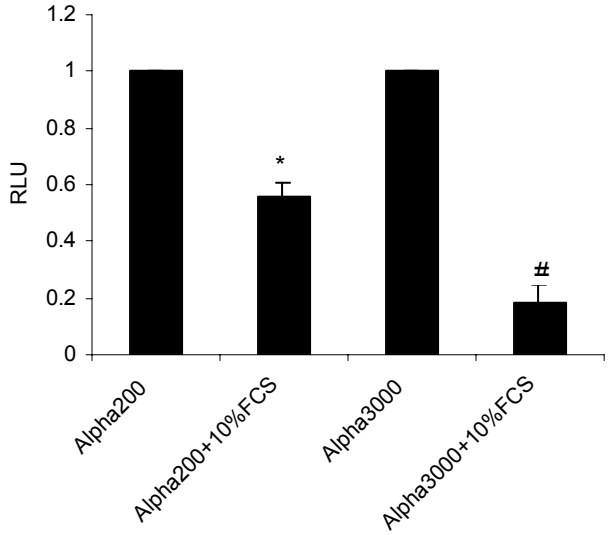


Figure 24: Effect of FCS on the activity of the truncated sGC α–1 promotor constructs in RLF-6 cells. RLF-6 cells were transfected with the sGC α–1 Alpha200 and Alpha3000 constructs and cultured without or with 10% FCS for 14-18 hours. Cell lysates were prepared and assayed for luciferase activity. Transfections were performed in triplicate. Results are in mean ± SD (n=3). *, # P<0.05; ANOVA.

The effect of 10% FCS on the promoter activity of the sGC α–1 gene was investigated by transfecting RFL-6 cells with the Alpha200 and Alpha3000 luciferase constructs and culture in the absence or presence of 10% FCS for 14-18 hours. Cell lysates were then prepared and assayed for luciferase activity. A clear reduction in the reporter gene expression of both Alpha200 and Alpha3000 constructs was observed upon FCS incubation (fig. 24). Thus, it was clear that FCS induced a down regulation of sGC α–1 by reducing its promoter activity.

3.4 Serum downregulation of sGC α-1 promoter is mediated by NFY and Sp1

In order to identify the transcription factors involved in the promoter regulation by FCS, an EMSA was performed with biotinylated NFY- and Sp1-specific oligodesoxynucleotides and the nuclear extract from RLF-6 cells cultured for 24 hours in the absence and presence of 10% FCS. There was an increased binding observed with the Sp1 probe (as indicated by the arrow), in contrast to a decreased binding with the NFY probe (Figure 31). Since the binding sites for NFY and Sp1 are overlapping in Alpha200, this implied that the presence of FCS increased the Sp1 binding and decreased the NFY binding to the site. Since we have observed that at this site, the binding of Sp1 leads to a decrease in promoter activity, hence we see a downregulation by FCS.

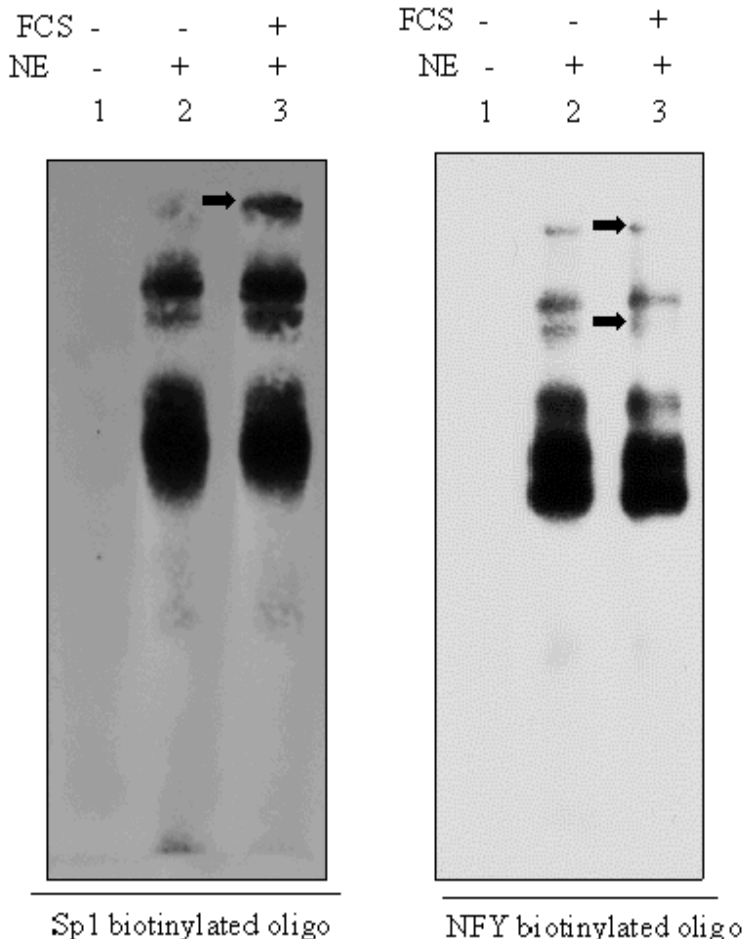


Figure 25: Effect of FCS on NFY and Sp1 transcription factor binding activity in nuclear extract of RFL-6 cells. 0.5 μg nuclear extract from RLF-6 cells cultured with or without 10% FCS for 24 hours were incubated with 2 ng of the biotinylated NFY and Sp1specific DNA probes. The complex were separated on a polyacrylamide gel and transferred onto nylon membranes. The detection of the biotin labelled DNA was carried out using the LightShift Chemiluminiscent EMSA Kit.

4 HuR mediated downregulation of sGC

The 3'-untranslated regions (UTR) of the sGC $\alpha-1$ and $\beta-1$ mRNA bear AU-rich elements (ARE), which are targeted by *trans*-acting factors for regulation of mRNA stability. One of these factors is the *elav*-like ubiquitous 34-kDa protein HuR, which binds to AREs, thereby protecting the respective mRNA from degradation (Fan XC, 1998a;Ma WJ, 1996). cGMP and cGMP-eliciting agents, such as YC-1, were found to decrease the expression of sGC $\alpha-1$ mRNA via down-regulation of HuR (Kloss S, 2003). In accordance, it was observed that exposure of isolated rat aortic segments to the activator of adenylyl cyclase, forskolin, strongly reduced sGC $\alpha-1/\beta-1$ and HuR protein and mRNA expression in a time-dependent and actinomycin D-sensitive fashion. Native protein extract from rat aorta shifted the electrophoretic mobility of biotin-labeled riboprobes from the 3'-untranslated region of sGC $\alpha-1$ and β-1 mRNA, and these bands was supershifted by a monoclonal antibody directed against HuR. Forskolin decreased the HuR- sGC $\alpha-1/\beta$-1 mRNA interaction and HuR protein expression in rat aorta, and this was prevented by the PKA inhibitory cAMP analog 3',5'-cyclic monophosphorothioate, Rp-isomer (Rp-cAMPS). In cultured smooth muscle cells from rat aorta, forskolin induced a rapid increase in Fos/p-Fos protein levels and activator protein 1 (AP-1) binding activity. To finally prove that activation of AP-1 accounts for cAMP-induced down-regulation of HuR, we used a transcription factor decoy approach.

4.1 Role of AP-1 in HuR mediated sGC downregulation

In general, cAMP signaling to the nucleus is accomplished by translocation of the catalytic subunit of PKA into the nucleus, where it phosphorylates and activates activating (cyclic nucleotide responsive element-binding protein) and silencing (inducible cAMP early repressor) transcription factors (Eigenthaler et al., 1999), both of which can target cyclic nucleotide responsive element sites, such as in

the *c-fos* promoter. It was observed that forskolin induced a very rapid increase in Fos and activated p-Fos that was accompanied by a transient activation of AP-1, the heterodimeric transcription factor constituted by Fos and Jun.

To prove that activation of AP-1 accounts for cAMP-induced down-regulation of HuR, we used a transcription factor decoy approach. RASMC starved for 24 hours were pretreated (4 hour) or not with either an AP-1-cognate oligodesoxynucleotide (match ODN) or a mutated ODN (mismatch), and then incubated for 6 hour with solvent (0.1% DMSO) or forskolin (10 μM). The cells were harvested, and HuR expression was assessed by Western blotting. As shown, the down-regulation of HuR by forskolin was prevented by the match AP-1 ODN, not by the mismatch ODN.

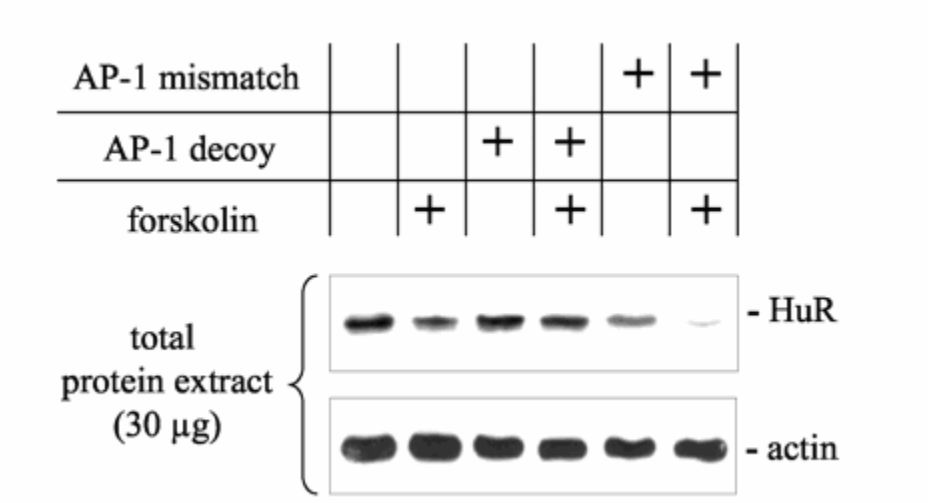


Figure 26. AP-1 decoy prevents forskolin-induced down-regulation of HuR in RASMC. Serum-starved (24 h) RASMC were exposed for 4 hours to 10 μM AP-1 oligodeoxynucleotide or a mutated ODN (mismatch, 10 μM), and then incubated for 6 hours with solvent (0.1% DMSO) or forskolin (10 μM). The cells were harvested, and HuR expression was assessed by Western blotting. The forskolin-induced decrease of HuR was prevented by AP-1 decoy, not by the mismatch ODN. Figure represents result from three experiments.

5 Identification of HuR Interacting Protein by Yeast Two Hybrid Analysis

While investigating the transcriptional regulation of sGC α-1 subunit expression, efforts were also made to study the post-transcriptional regulation of sGC by the mRNA stabilizing protein, HuR. It has been established that HuR stabilises the sGC α–1 and β-1 mRNA. However the pathway underlying this regulation remains unknown. In order to identify the mechanism of this regulation, we looked for HuR interacting proteins employing the yeast two hybrid assay. The yeast two-hybrid system is devised to identify genes encoding proteins that are physically associated with a given protein in vivo. The LexA Two-Hybrid System was used in this study wherein the DNA-BD (DNA-binding domain) is provided by the entire prokaryotic LexA protein, which normally functions as a repressor of SOS genes in *E. coli* when it binds to LexA operators. The AD (activation domain) is an 88-residue acidic *E. coli* peptide (B42) that activates transcription in yeast. Two different cloning vectors are used to generate fusions of these domains to genes encoding proteins that potentially interact with each other, and the recombinant hybrid proteins are coexpressed in yeast. An interaction between a target protein (fused to the DNABD) and a library-encoded protein (fused to the AD) creates a novel transcriptional activator with binding affinity for LexA operators. This factor then activates reporter genes having upstream LexA operators and this makes the protein-protein interaction phenotypically detectable. If the two hybrid proteins do not interact with each other, the reporter genes will not be transcibed. As a confirmation of the interaction outside the yeast cell, several assays are used, such as in vitro "pull-down" assays and co-immunoprecipitation.

5.1 Construction of the "bait"

To construct the LexA-fused bait protein, we amplified human HuR cDNA (corresponding to residues 1-326 of the protein sequence) by polymerase chain reaction (PCR) using forward primer 5'-*gcgaattcatgtctaatggttatgaa*-3' with a flanking *EcoRI* restriction site and reverse primer 5'-*gctctcgagttatttgtgggactt*-3' with a flanking *XhoI* restriction site. pGEX-HuR was obtained from Dr. Stephan Kloess in our laboratory and was used as the template for HuR. The thermal cycling programme was followed as mentioned in Methods section; the annealing temperature was 50^0C for 45 seconds and the extension was carried out for 1 minute at 70^0C. The fragment was first subcloned into pBSKS (fig. 27), verified for the sequence and finally inserted into the multiple cloning site into the *EcoRI* and *XhoI* restriction sites of pLexA (pLexA-HuR_{1-326}). The yeast-two hybrid screening was carried out by using a human placenta cDNA library in the vector pJG4-5.

	DB vector selection marker (pLexA)	AD vector selection marker (pJG4-5)	Chromosomal reporter gene	Plasmid reporter gene
LexA System	*HIS3*	*TRP1*	*LEU2*	*LacZ*

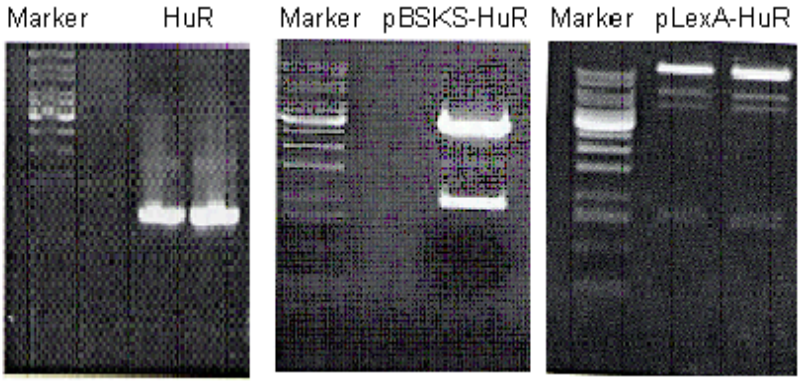


Figure 27: Construction of the "bait". HuR was generated by PCR using pGEX-HuR as the template and purified by gel extraction. The fragment was first subcloned into pBSKS and verified for the sequence. The sequenced fragment was finally cloned into pLexA.

5.2 HuR expression in yeast

Before proceeding with screening, the actual expression of the fusion protein inside yeast was verified by SDS-PAGE and subsequent western blot analysis. After transformation of the plasmids encoding the fusion protein into *Saccharomyces cerevisiae* strain EGY48 and incubation on plates lacking the appropriate amino acids (e.g.Trp), single colonies were inoculated into 15 ml synthetic medium lacking the same amino acid(s). At an optical density (A_{600}) around 0.7, the culture was centrifuged at 2500 rpm for 5 minutes; the pellet was washed in distilled water and boiled for 3 minutes in 200 µl of 4x Roti load buffer. 50 µl of this sample was separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Detection of the expressed fusion proteins was performed with polyclonal anti-LexA antibody and peroxidase-conjugated anti-rabbit antibody using ECL (Amersham Life Science, Amersham, U.K.). The results showed that HuR was being expressed in the yeast cells (fig. 28).

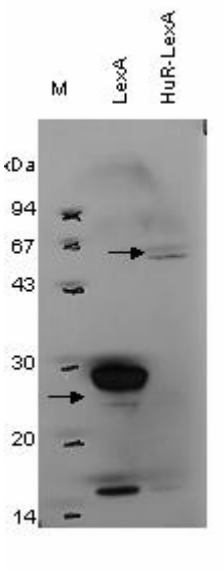


Figure 28: Western Blot for HuR expression in EGY48. pLexA and pLexA-HuR were transformed separately in yeast cells and the lysate was investigated for HuR expression using anti-LexA antibody (1:5000) and peroxidase-conjugated anti-rabbit antibody (1:10000). Arrows indicate LexA (30 kDa) and HuR-LexA (62 kDa). M denotes the marker profile.

5.3 Auto-activation

Since the yeast two-hybrid system is based on reconstitution of a functional transcription factor, checking the auto-activation capacity of the target is crucial for the overall feasibility. To check for the auto-activation, the yeast interaction trap assay was carried out with pLexA-HuR_{1-326} and the empty activation domain vector pJG4-5. No growth and blue coloring was observed on minimal agar plates lacking uracil, histidine, tryptophan, and leucine, supplemented with 2% galactose, 1% raffinose, and 80 μg/ml 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal) indicating that there was no auto-activation.

5.4 Yeast-two hybrid screening

Plasmids expressing human HuR fused to the LexA DNA binding domain were introduced in yeast strain EGY48. EGY48 containing pSH18–34 and pLexA-HuR_{1-326} was transformed with the human placenta Matchmaker cDNA library and ~1 x 10^7 transformants were plated on selection medium (leu2). Colonies were screened by the ß-galactosidase reporter gene assay (lacZ) using X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) plates.

Library plasmids of positive yeast clones (blue spots) were rescued by transformation of *Escherichia coli* KC8 strain. Generally, 3 colonies were picked up from each transformation and inoculated in LB-broth with ampicillin for miniprep of plasmids. Plasmid preparations obtained from the bacterial cultures were tested for the presence of the vector pJG4-5 containing the insert by restriction enzyme digests (fig. 29A & B). The resulting pattern of restriction fragments on the electrophoresis gel was compared to the pattern obtained by the digestion of the empty AD vector.

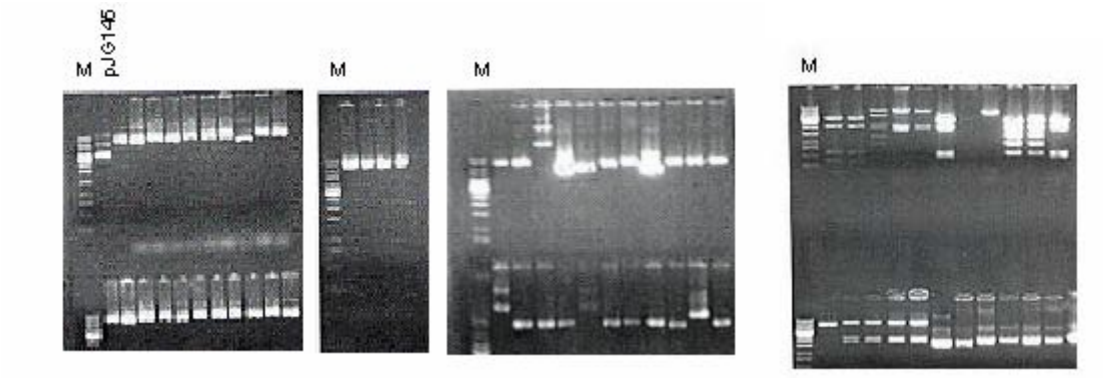


Figure 29A: Screening of plasmids for the presence of inserts in pJG145. DNA obtained from positive coloured (blue) yeast colonies were transformed into *E.coli KC8*, plasmids were isolated and the DNA was digested with HindIII. Restriction fragment pattern was compared with the pattern of pJG145 digestion for the presence of inserts. M denotes the marker profile.

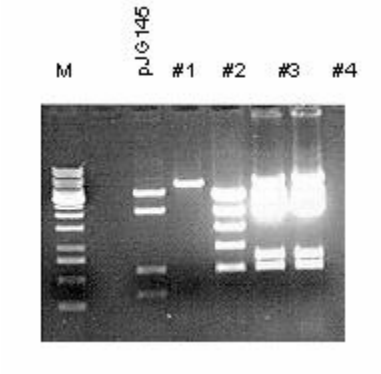


Figure 29B: Restriction digestion of plasmids selected from the above screening. 4 plasmids isolated from the yeast colonies showing a different pattern from pJG145 digestion were selected and tested again. From these, clones #2 and #4 were sent for sequencing for their identity. M denotes the marker profile.

To test the specificity of protein–protein interaction, EGY48/pSH18–34 was retransformed with the rescued library plasmids. The yeast reporter strain contained pLexA-HuR$_{1–326}$ or empty pLexA as the bait. Transformants were assayed for *leu2* and *lacZ* activity to reconfirm the interaction. Upon retransformation, only colonies from #2 and #4 turned out to be positive in terms of growth and blue coloration on minimal agar plates lacking uracil, histidine, tryptophan, and leucine, supplemented with 2% galactose, 1% raffinose, and 80 μg/ml 5-bromo-4-chloro-3-indolyl--D-galactopyranoside (X-gal). DNA sequences of the two plasmids were determined by the dideoxy chain termination method (MWG Biotech, Ebersberg, Germany). #2 was identified as *Homo sapiens* spermidine/spermine N1-acetyltransferase (SSAT) mRNA (accession No. NM_002970), #4 turned out to be a part of a vector that might have been used during the construction of the placenta library.

5.5 Immunoprecipitation

As a means to reconfirm the interaction by other approaches, immunoprecipitation was performed as mentioned in 2.23. Lysate of COS-1 endogenously expressing HuR was prepared and 2 - 5 μl of the anti-rabbit antibody was added as the control and anti-SSAT antibody was added for immunoprecipitation. Western blot was performed using a monoclonal mouse anti-HuR antibody as the primary (1:400) and anti-mouse antibody (1:5000) as the secondary antibody.

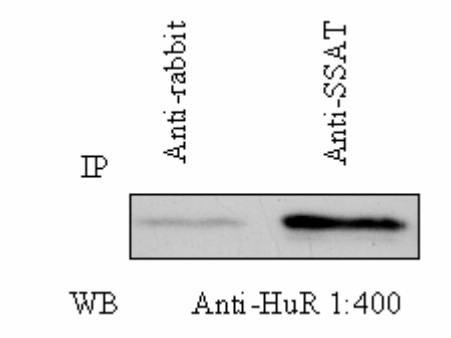


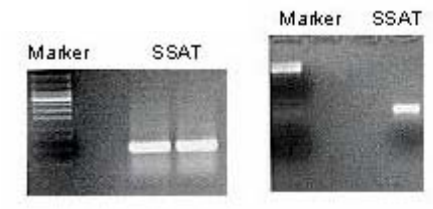
Figure 30: Immunoprecipitation assay. Immunoprecipitation (IP) from lysates of COS-1 cells endogenously expressing HuR, using anti-SSAT antibody. Western blotting (WB) was done with anti-HuR antibody (left). For control, anti-rabbit antibody was used.

The results clearly showed that SSAT and HuR were interacting at the *in-vivo* level; a strong HuR band was detected with anti-SSAT antibody as compared to the control. The anti-SSAT antibody was binding to SSAT which in turn interacted with HuR seen as a band in fig. 30.

5.6 Construction of expression plasmids for SSAT

For bacterial expression of identified interaction partner as GST fusion proteins, we amplified human SSAT cDNA by polymerase chain reaction (PCR) using forward primer 5'-*gcgaattcatggctaaattcgtgatccg*-3' with a flanking *EcoRI* restriction site and reverse primer 5'-*gctctcgagtcactcctctgttgcc*-3' with a flanking *XhoI* restriction site using pJG4-5-SSAT as the template. The thermal cycling programme was followed as mentioned in Methods section; the annealing temperature was 56^0C for 45 seconds and the extension was carried out for 90 seconds at 70^0C. The fragment was first subcloned into pBSKS (fig. 31), verified for the sequence and finally excised by *EcoRI* and *XhoI* and ligated into pGEX previously modified for in-frame expression of *EcoRI/XhoI* fragments. *E. coli* BL21 strain was transformed with recombinant pGEX plasmids, and GST fusion proteins from exponentially growing bacteria were purified by GSH-sepharose affinity chromatography according to the manufacturer's instructions (Pharmacia).

A)



B)

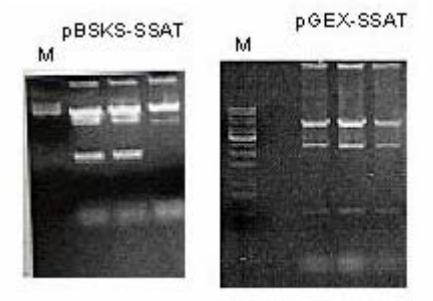


Figure 31: Construction of Expression Plasmids for SSAT. A) SSAT was generated by PCR using pJG145-SSAT as the template and purified by gel extraction. **B)** The fragment was first subcloned into pBSKS and verified for the sequence. The sequenced fragment was finally cloned into pGEX. M denotes the marker profile.

5.7 Confirmation of SSAT-HuR interaction by GST-pull-down

Another method to confirm the SSAT-HuR interaction was through GST-pulldown which was carried out as mentioned in 2.25. To the cell lysate from pGEX-SSAT transfected COS-1 cells, glutathione sepharose 4B beads loaded with the GST fusion protein was added. Western blot was performed and the protein was detected with anti-HuR antibody (1:400) and anti-mouse antibody (1:5000).

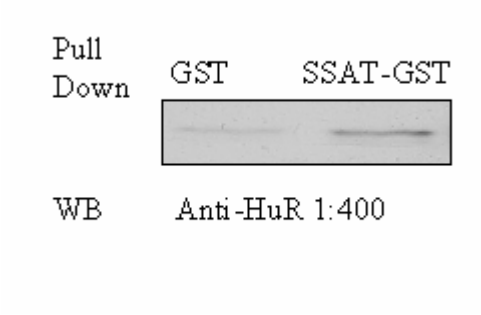


Figure 32: GST pulldown assay. GST-pulldown assay was performed with lysates of COS-1 cells transfected with SSAT-GST. Western blotting was done with anti-HuR antibody. For control, GST expressing cell lysate was used.

This was an in-vitro approach to detect the interaction. The presence of a strong HuR band in the blot as compared to the control lane (fig. 32) clearly suggested that the interaction between HuR and SSAT happened in-vitro also.

5.8 Preparation of deletion mutants of HuR

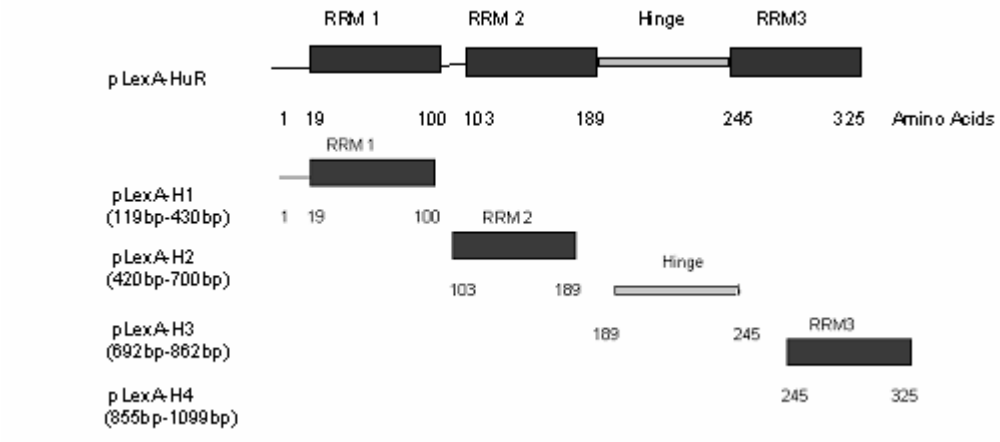


Figure 33: Deletion mutants of HuR. The deletion constructs were cloned into pEG202/pLexA vectors. The size of the regions in terms of the number of amino acids and base pairs is shown by black (RRM1,2,3) and grey (Hinge) bars, respectively. The constructs were named pLexA-H1 through H4.

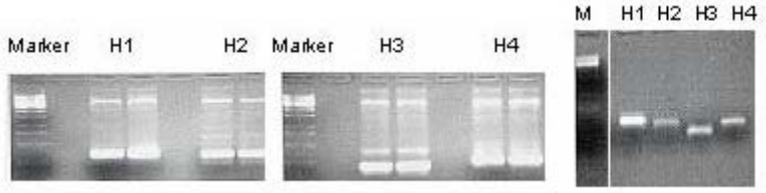
HuR can be divided into three RNA recognition motifs (RRM 1, 2 and 3), and one hinge region (fig. 33). Thus four constructs having each of them separately were generated from PCR using the following primers (Table 8):

Table 8: List of the primers used for the PCR amplification of H1, H2, H3 and H4 regions of HuR.

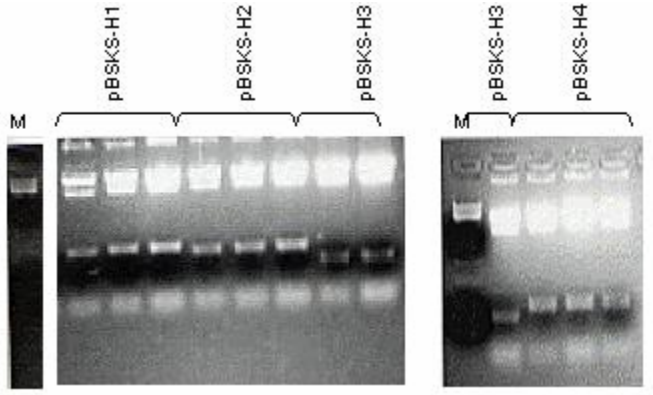
For H1:	Forward - *gcgaattcatgtctaatggttatgaa* Reverse - *gctctcgagtcatttgatcacctctgagc*
For H2:	Forward - *gcgaattcatggaggtgatcaaagacgccaac* Reverse - *gctctcgagtcacacgtttttgttctgg*
For H3:	Forward - *gcgaattcatgaacaaaaacgtggcactcc* Reverse - *gctctcgagtcagatgaaaatgcaccagcc*
For H4:	Forward - *gcgaattcatgattttcatctacaacctggg* Reverse - *gctctcgagttatttgtgggacttg*

All the forward primers had a flanking *EcoRI* restriction site and reverse primers had a flanking *XhoI* restriction site. pLexA-HuR was used as the template. The thermal cycling programme was followed as mentioned in Methods section; the annealing temperature was 56°C for 45 seconds and the extension was carried out for 1 minute at 70°C. The fragments were first subcloned into pBSKS verified for their sequence and finally excised by *EcoRI* and *XhoI* and ligated into pLexA (fig. 34).

A)



B)



C)

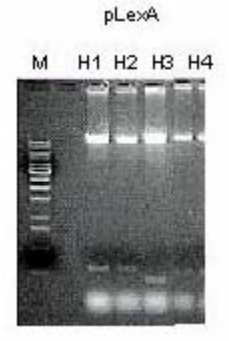


Figure 34: Generation of deletion mutants of HuR. A) H1-H4 were generated by PCR using pLexA HuR as the template and purified by gel extraction. **B)** The fragments were first subcloned into pBSKS and verified for their sequence. **C)** The sequenced fragments were finally cloned into pLexA. M denotes the marker profile.

5.9 Mapping of the interacting domain of HuR

For mapping the domains of HuR which interact with SSAT, yeast trap interaction assay was carried out as mentioned in the Methods section. pJG4-5-SSAT and the different deletion constructs were transformed separately in the opposite strains of yeast. Cell suspensions from each (50 μl each) were mixed and incubated in rich medium (YPD) at 30^0C for 14-16 hours. Interactions were validated by growth and blue coloring on minimal agar plates containing X-gal.

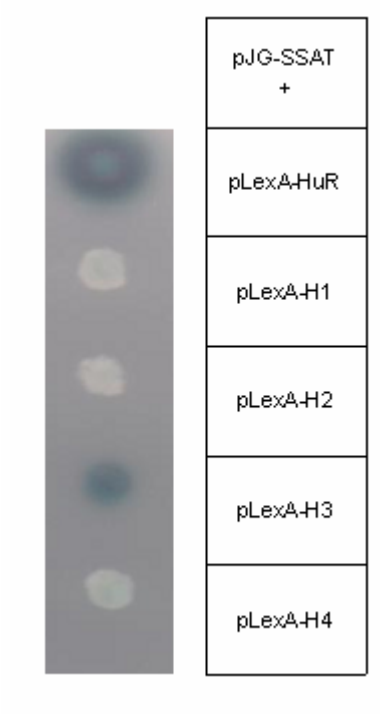


Figure 35: Mapping of the interacting domain of HuR. EGY48/pSH18–34 was transformed with pJG-SSAT and full length or the deletion constructs of HuR and yeast interaction trap assay was performed. Interactions were validated by growth and blue coloring on minimal agar plates lacking uracil, histidine, tryptophan, and leucine, supplemented with 2% galactose, 1% raffinose, and 80 μg/ml 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal).

The suspension carrying pJG4-5-SSAT and pLexA-H3 only turned blue whereas all the other three combinations of pJG4-5-SSAT with H1, H2 and H4 remained white (fig. 35). This clearly showed that only H3 region of HuR interacted with SSAT. H3 region of HuR is its hinge region (Fan XC, 1998b).

5.10 Influence of HuR on SSAT activity

In order to characterize the functional implications of the HuR-SSAT interaction, studies were performed to check if they affected each other's biological activity. SSAT is an enzyme of the catabolic pathway that acetylates spermine/spermidine. The activity of the enzyme was measured either alone or in combination of HuR through the SSAT activity assay, which measures the amount of ^{14}C-acetylated-spermidine. As shown in figure, HuR did not affect SSAT activity.

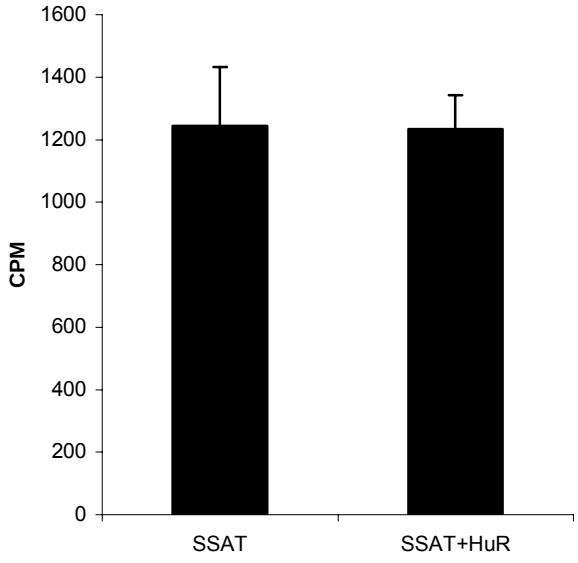


Figure 36. Effect of HuR on SSAT activity. The SSAT activity assay was carried out with 250 ng of SSAT-GST, 30 mM spermidine and 0.5 µL [^{14}C]Acetyl Coenzyme A in the presence or absence of 250 ng of HuR. The reaction was stopped; the mixture was put on Whatman paper filters, dried and washed. The filters were then placed in the scintillation fluid and counted. The figure represents results from three independent experiments.

5.11 Effect of SSAT on HuR binding to mRNA

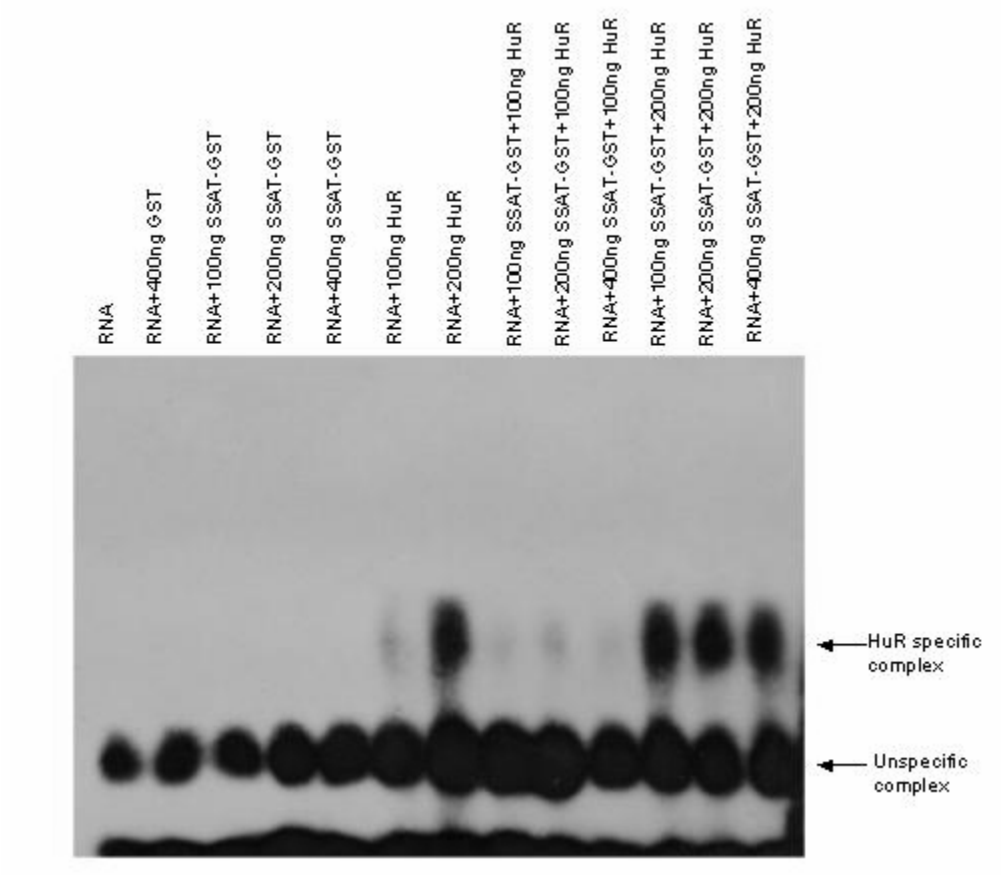


Figure 37. Effect of SSAT on sGC-mRNA-binding activity of HuR. Biotinylated oligoribonucleotide from the 3'UTR of GC A-1 mRNA (DR_1GC3UTR2; 7.5 ng) was incubated for 30 minutes at 4°C with HuR in the presence or absence of 100-400 ng of SSAT-GST, and the EMSA was performed using an 8% TAE-polyacrylamide gel. Only GST was used as control for SSAT-GST. Figure represents result from three experiments.

HuR is a ubiquitously expressed RNA binding protein that has been found to regulate the expression of sGC α-1 by binding to its 3'UTR mRNA. Since SSAT has been shown to interact with HuR, we performed an EMSA to see whether the HuR binding to sGC α-1 mRNA is affected in the presence of SSAT. Biotinylated oligoribonucleotide from the 3'UTR of sGC α-1 mRNA (comprising several AREs) was incubated with HuR (100 and 200 ng) with increasing concentrations of

SSAT-GST (100-400 ng), and the mixture was analyzed by RNA-EMSA as described in Methods. HuR (200 ng) induced a prominent bandshift of the free riboprobe (fig. 37). The intensity of this shifted band was not at all afffected by the presence of SSAT (fig.37). Therefore, this finding demonstrates that SSAT does not affect the binding of HuR to the 3-UTR of sGC α-1 mRNA.

IV Discussion

Soluble guanylyl cyclase (sGC) is a key signaling component of the L-arginine-NO-cyclic GMP pathway (Bellamy TC, 2002);(Denninger JW, 1999). The propagation of NO signaling via cyclic GMP formation is influenced at different time scales, most rapidly by allosteric activation of heterodimeric sGC upon binding of NO to its heme-iron. In addition, NO signaling may be affected by altered expression of sGC subunits, as has been shown in different pathological conditions (Kloss S, 2000);(Li D, 1999;Marques M, 2001);(Mulsch A, 2001);(Takata M, 2001);(Telfer JF, 2001);(Tzao C, 2001) and developmental stages (Behrends S, 2001);(Bloch KD, 1997);(Giuili G, 1994);(Ibarra C, 2001);(White CR, 2000). The molecular mechanisms underlying altered sGC expression in these and other conditions have not yet been revealed.

Elevation of intracellular cGMP can decrease mRNA levels of sGC α-1 and β-1 subunits, iNOS, COX-2, TNF-α, and TGF-β_3 via message destabilization in VSMCs, mesangial cells, and cardiac fibroblasts (Alexandra K.Kiemer and Angelika M.Vollmar, 1998);(Dolores Pérez-Sala, 2001);(Filippov G, 1997);(Kloss S, 2003);(Nadia Abdelaziz, 2001);(Sinha B, 1998). Stability of sGC α-1 mRNA is regulated by the ubiquitous mRNA binding protein HuR, which binds to AU-rich elements in the 3'-untranslated region (UTR) and increases mRNA half-life; cGMP-elevating agents decrease expression and RNA binding of HuR, thereby destabilizing sGC α-1 mRNA (Kloss S, 2003). The cGMP-induced mRNA destabilization requires transcription of an unknown factor(s) (Kloss S, 2003);(Nadia Abdelaziz, 2001); this could be an RNA-destabilizing protein or a factor involved in downregulation of HuR.

This study was performed in an effort to provide some insight to the transcriptional and post-transcriptional regulation of sGC expression in a mammal, the rat. The sGC α-1 subunit promoter was analysed for the regulation of gene expression and the responsible transcription factors were identified.

Evidence of sGC regulation at the transcriptional level in response to a stimulus was observed and the possible mechanism was outlined. An effort was made to characterize the molecule affecting the stability of sGC mRNA.

1 Influence of genomic organization on transcriptional regulation of sGC subunits within different species

Comparison of the genomic organization of α-1 and β-1 sGC genes between rat, Medaka fish, mouse, and human (Azam M, 1998);(Giuili G, 1993);(Sharina IG, 2000);(Yao Y, 2003) (species where the sGC genomic organization was resolved) demonstrated several interesting features. The mammalian α-1 and β-1 sGC genes are further from each other on the chromosome than the Medaka fish genes. In the latter species they are separated by only 1 kb (Mikami T, 1999), suggesting a concerted regulation of expression of both subunits. Indeed, tandem genomic organization relates to a directly coordinated transcription for α-1 and β-1 subunits in Medaka fish (Mikami T, 1999), however, this option is topologically excluded in mammals. The mouse α-1 and β-1 sGC genes map to the third chromosome. However, they are separated from each other by an extended region comprising 2% of the total chromosomal length. Probing of the Human Genome Database with the mouse and human cDNA sequences identified the clone AC021433, which contains eight exons of the human α-1 gene and 14 exons of the human β-1 gene. This fragment is ascribed to human chromosome 2. The rat α-1 and β-1 genes map to chromosome 2.

The transcriptional activity of promoters for mouse α-1 and β-1, and human $\beta-1$ genes have also been demonstrated to be different (Sharina IG, 2000). Independent transcription of different sGC subunits existing in mammals could be beneficial in tissue-specific regulation of expression, which was speculated to be one mechanism of regulation of sGC activity (ref. (Andreopoulos S, 2000) and references therein).

The transcriptional regulation of the expression of sGC has not been previously examined. Recently, evidence to support altered expression of mRNA of sGC subunits has emerged. In primary rat pulmonary artery smooth muscle cells, prolonged NO treatment leads to decreased NO-stimulated sGC activity and mRNA levels (Filippov G, 1997). sGC levels rise in unborn rat pulmonary artery, beginning at approximately 20 days of gestation, and mRNA, protein, and activity remain elevated at least 8 days after birth (Bloch KD, 1997). Decreased rates of sGC transcription also have been suggested in other models after NO treatment and administration of cAMP-elevating agents (Behrends S, 1995);(Chhajlani V, 1991). Furthermore, nerve growth factor administration to rat PC-12 cells results in decreased steady-state levels of sGC α–1 and β–1 mRNA (Liu H, 1997). Estrogen treatment has been reported to decrease α–1 and β–1 sGC mRNA levels in rat uterus (Krumenacker JS, 2001). However the precise mechanisms underlying these effects on sGC in specific tissues are largely unknown. Our findings on the activity of putative promoter regions demonstrate basal transcriptional activity for sGC α-1 subunit. These data represent the first studies on the transcriptional regulation of rat sGC genes and will help to gain insights on the genetic basis of regulation of this important enzyme.

2 Basal activity of sGC α-1 promoter

The studies performed by Dr. Meik Behrens showed that the sGC subunits in rats are also independently regulated. GenomeWalking from the 5' UTR of the α-1 subunit resulted in different clones that were subsequently sequenced and cloned into the pGL3basic vector (Invitrogen) containing luciferase as reporter gene. For the α-1 subunit following clones were obtained Alpha3000 (with -2794 bp), Alpha1100 (-1092 bp), Alpha350 (-346 bp) and Alpha200 (-200 bp) (see fig. 1). Transfection of the constructs of both subunits in rat vascular smooth muscle cells resulted for the Alpha3000 construct in a 8.4$\pm$2.2 fold, the Alpha1100 a 3.2$\pm$fold, Alpha350 a 15.1$\pm$1.8 fold and Alpha200 a 28.4$\pm$4.7 fold induction

compared to the empty vector (Fig. 32). The Alpha200 construct containing ~200bp upstream showed highest level of activity. The changes in the activity among Alpha200, Alpha350, Alpha1100 would indicate the presence of a repressor(s) between –200 and -3000 bp, and an enhancer element(s) located 200 bp upstream of the transcriptional start site. The 1100 bp deletion caused a 5-fold decrease in activity, suggesting that putative enhancers were located in between –3000 and –1100 bp and –1100 and –350 bp. Since the promoter activity was the highest in the Alpha200 construct, it suggested that this fragment contains all the crucial elements necessary to support basal transcription of the sGCα_1 gene. Hence Alpha200 was chosen to validate the results in RLF-6 cells. Promoter analysis in cultured rat aortic smooth muscle cells was hampered by the low efficiency of transfection wih the promotor-luciferase constructs, which would have made it difficult to observe changes in promoter activity in response to various stimuli. Since the RLF-6 cells were found to give five-fold higher signals as compared to the primary smooth muscle cells, the RLF-6 were used for subsequent experiments. Another advantage of these cells as a model to investigate transcriptional activity of sGC was that this cell line consistently expresses high levels of the $\alpha-1/\beta-1$ sGC heterodimer. Transiently transfected luciferase reporter constructs harboring 0.2 and 3.0 kb of α-1 sGC upstream sequence were analysed for promoter activity (Fig. 3). As expected, Alpha200 construct resulted in a 6.02+-0.41-fold induction as compared to Alpha3000 construct. Since the promoter activity was the highest in the 0.2 kb construct, it suggested that this fragment contains all the crucial elements necessary to support basal transcription of the sGC α-1 gene.

Functional analysis of constructs containing different size fragments of the 5'-flanking region demonstrated a variable profile of activity, suggesting the presence of several enhancers and repressors functioning at a distance from the regulatory promoter. The Alpha200 construct demonstrated the highest activity, indicating that the regulatory promoter of the α-1 sGC encompasses ~200 bp preceding the transcriptional start site. In the present report, we focused on

characterization of the promoter and identification of the transcriptional factors responsible for the constitutive transcription of a_1 sGC in RLF-6 cells. The α-1 sGC gene possesses a TATA-less promoter. Mouse α-1 promoter and human β-1 promoter for sGC which have been analysed have been both found to be TATAless promoters (Sharina IG, 2003);(Vazquez-Padron RI, 2004). Detailed sequence analysis revealed several putative binding sites in the immediate proximity of the start site, which were subsequently analysed.

3 NFY and Sp1 are responsible for the basal expression of sGC α-1 in RLF-6 cells

Analysis of the 5' UTR of the rat α-1 sGC gene revealed a multiplicity of putative transcription factor binding sites that seemed to be clustered along the promoter region. The presence of multiple copies of putative binding sites for transcriptional factors involved in hematopoietic development and differentiation, such as GATA1, LMO2, IK2 (Schmitt C, 2002), (Wadman IA, 1997), and factors involved in morphogenesis and development, such as Sox5, Prx2 and Nkx-2.5 (Jamali M, 2001);(Smits P, 2003);(ten Berge D, 2001), is noteworthy. The relevance of this observation is substantiated by evidence that sGC activity is important during embryogenesis (Bloch KD, 1997);(Harumi T, 2003);(Smigrodzki R, 1996);(Yamamoto T, 2003). However, it would require further studies to address the mechanism of α–1 sGC transcriptional regulation during development and hematopoiesis.

The functional significance of consensus transcriptional factor binding sites proximal to the transcriptional start site was investigated by decoy oligonucleotide approach and site deletions in the full length and the 200-bp promoter fragment. The elimination of CCAAT-binding factor (NFY) binding core significantly diminished whereas deletion of the Sp1 core elevated the transcription rate (fig. 14). Electrophoretic mobility-shift assay (EMSA) confirmed the interaction of NFY, and Sp1 factors with the sGC α-1 promoter (figs. 18 and 20). Treatment of RLF-6

cells with genistein and mithramycin A, known to inhibit the NFY binding and Sp1 binding to DNA respectively, significantly reduced protein levels of sGC α-1 by inhibiting transcription (figs. 16, 17 and 21). In summary, our study represents an analysis of the rat sGC α-1 promoter regulation in RLF-6 cells and identifies NFY and Sp1 as critically important factors in sGC α-1 expression

Core deletion of the sites caused a markedly visible reduction in the activity of both the Alpha200 and the Alpha3000 constructs (fig. 14), emphasising the significance of these sites in the promoter activity. Also it became clear that Sp1 is indeed an activating element for both the long and the short fragments. The use of decoy ODN and chemical inhibitors are indirect approaches and could lead to diverse changes in the cell so that the results observed in those cases can be contributed to that effect.

As shown in fig. 19 the abundance of the targeted transcription factors were reduced in the cells treated with the corresponding decoy ODN. Transfection with the transcription factors CABL and MZF decoy ODN had no effect on the promoter activity (fig. 11). In contrast, the NFY-specific decoy resulted in a significant decrease, indicating that NFY could function as an enhancing element in basal sGC α−1 core promotor activity. The Sp1-specific decoy resulted in an increase of the promoter activity, which could mean that this factor might be involved in a negative regulation of sGC α−1 transcription.

Treatment of RLF-6 cells withinhibitors of the transcription factors NFY and Sp1 confirmed the results from the decoy experiment and established NFY as the activating and Sp1 as the repressing element in the 200 bp construct. Also it clearly demonstrated that the effects of mithramycin A on the Alpha200 and the Alpha3000 construct are completely opposite. This finding could mean that Sp1 acted as a repressor for the site present in the 200 bp region whereas its effect was to activate when it was bound to the other sites in the long construct.

With EMSA, major and minor complexes were detected in the case of both the oligos (figs. 18, 19 and 20). Several transcription factors are able to bind the CCAAT consensus sequence; among them are NFY-A, B and C subunits, likewise Sp1, 2, 3 and 4 for the Sp1 consensus sequence. To identify the transcriptional factors bound to the respective probes, we performed a supershift analysis by using antibodies dirtected against these putative transcription factors. With the CCAAT probe, a supershift was achieved with an antibody specific for NFY-B. The presence of NFY-A antibody did not produce a supershift, however, it induced a decrease of band intensity in the formed complexes, suggesting that formation of protein/DNA complexes with the probe could depend on the availability of this factor. With the Sp1 probe, antibody against Sp1 led to a shift of the complex. These results indicated that NFY-B and Sp1 are the major components of the protein complexes bound to the CCAAT and the Sp1 probes respectively.

The CCAAT box is one of the most common elements in eukaryotic promoters, found in the forward or reverse orientation (Mantovani R., 1999). One of the various DNA binding proteins that interact with this sequence is NFY. NFY consists of three different subunits, NFY-A, NFY-B, and NFY-C, that all are necessary to form a NFY-DNA complex (Hooft van Huijsduijnen R, 1990);(Maity SN, 1992);(Maity SN, 1990);(Sinha S, 1995);(Vuorio T, 1990). Specific amino acid segments in each NFY subunit are highly conserved during evolution. Only NFY (NFY, HAP2/3/4/5) has been shown to absolutely require all 5 nt. The frequency of CCAAT boxes appears to be relatively higher in TATA-less promoters, particularly in the reverse ATTGG orientation. The sGC α-1 promoter has the CCAAT box in the reverse orientation and is a TATA-less promoter. In TATA-containing promoters the CCAAT box is preferentially located in the -80/-100 region (mean position -89) and is not found nearer to the Start site than -50. In TATA-less promoters it is usually closer to the +1 signal (at -66 on average). The rat sGC $\alpha-1$ has the CCAAT box at –65. Similarly, the promoter region of human sGC $\beta-1$, which is also TATA-less, has the CCAAT box at –50 in the

reverse orientation. This box is essential for the basal promoter activity (Sharina IG, 2003).

Elevated levels of sGC expression and high cGMP content were detected in human bladder carcinoma in comparison with normal tissue (Ehsan A, 2002). It has also been recognized that tumors have altered guanylyl cyclase activity and that tumor bearing animals have elevated cGMP urinary excretion (Murad F, 1975), (Kimura H, 1975b). Conversely, genistein, has been found to inhibit the growth of carcinogen-induced cancers in rats and human leukemia cells transplanted into mice (Zhou JR, 1998). Since we found that genistein inhibited the expression of sGC α–1 (fig. 15) it is temptive to speculate that the anti-cancer activity of genistein may be related to its ability to reduce the level of sGC α–1 expression and consequently NO-dependent generation of cGMP by sGC.

Sp1 was originally characterized as a transcription factor that recognized GC-rich sequences in the early promoter of Simian Virus 40 (Dynan WS, 1983);(Gidoni D, 1984). Sp1 is considered to be a constitutively expressed transcription factor and has been implicated in the regulation of a wide variety of housekeeping genes, genes that are expressed in a tissue-specific manner, and genes involved in the regulation of growth such as in cell cycle or mammalian development (Hagen G, 1994). The embryos of Sp1-null mice exhibit severely retarded growth and die after day 10 of embryonic development (E10) (Marin M, 1997). Thus, it is likely that Sp1 is essential for the differentiation of embryonic stem (ES) cells after day 10 of gestation. Sp1 is one of the first cellular transcription factors to be identified, cloned and characterized for its ability to bind to GC-boxes in regulatory promoter elements. It belongs to the Sp family of transcription factors that currently includes four proteins designated Sp1, Sp2, Sp3 and Sp4 (Suske G., 1999).

Sp1 interacts with many factors, such as the TATA-box-binding protein (TBP), which is a major component of the general transcriptional machinery, as well as

with the TBP-associated factors dTAFII110 (Hagen G, 1994), hTAFII130 (Tanese N, 1996), and hTAFII55 (Chiang CM, 1995). Other proteins, such as transcription factors YY1 (Lee JS, 1993),(Seto E, 1993), E2F (Karlseder J, 1996);(Lin SY, 1996) and co-activators p300/CBP (Billon N, 1999);(Xiao H, 2000) and CRSP (cofactor required for Sp1 activation) (Naar AM, 2002);(Ryu S, 1999);(Taatjes DJ, 2002), have also been reported to associate with Sp1. A specific protein-protein interaction between the glutamine-rich region of Sp1 and hTAFII130 is required for recruitment of general transcription factors (Dunah AW, 2002).

Sp1 and NFY have also been shown to interact (Roder K, 1999), which can play an important role, in regulation of promoters where both transcription factors have overlapping binding sites. Examples are the Sp1 gene promotor itself, the major histocompatibility complex class II-associated invariant chain, and sGC α–1 gene promoter (Nicolas M, 2003);(Wright KL, 1995). Analysis of the Sp1 gene promoter showed that both factors compete for the NFY/Sp1 overlapping box whereas in the MHC invariant chain promoter the expression was upregulated by cooperative interactions of the factors. The sGC α–1 promoter has the overlapping NFY/Sp1 region at bases –60 to –65 from transcription start. Our experiments performed to analyse this promotor region suggested that NFY had an activating and Sp1 had a silencing effect on sGC α–1 gene expression. It is possible that the regulation of the sGC α–1 promoter depends on the interaction between both factors. This interaction could be either synergistic or competitive. Also the interaction between both factors might change in response to an external stimulus, leading to an up- or downregulation of sGC α–1 gene expression.

4 Serum downregulates the sGC α-1 Expression in RLF-6 cells

When we exposed serum-starved RLF-6 cells or freshly isolated rat aorta to 10 % fetal calf serum for 24 hours and then analysed the cells and tissue for expression of sGC α−1 we observed a marked down-regulation at the mRNA and protein level (figs. 22 and 23). In consistency with the above observations, 10% FCS also led to a decrease in the activity of the sGC α−1 promoter, as assessed by luciferase reporter assay (fig. 24).

Proliferation of vascular smooth muscle cells (VSMCs) is an important pathological event in a number of vascular disease processes including atherosclerosis, the response to endothelial injury, vascular rejection, hypertension or normal aging (Laurent S, 1996);(Pilz RB, 2003). Serum has been shown to have a mitogenic effect on the proliferation of smooth muscle cells. By studying the growth patterns of VSMC from spontaneously hypertensive rats (SHRs) vs. those obtained from their normotensive counterparts, Wistar-Kyoto (WKY) rats, it was demonstrated that VSMC from SHRs exhibited a higher specific growth rate, abnormal contact inhibition, and accelerated entry into the S phase of the cell cycle (Kloss S, 2000). Moreover, they were hyperresponsive to many growth factors such as calf serum, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor beta 1 (TGF beta 1), and insulin. These intrinsic growth anomalies in cells of hypertensive origin persist in culture indicating their putative primary role in the pathogenesis of hypertension (Ghosh J, 2005);(Mikhail N, 1993). In context of these observations, serum has also been found to up-regulate the expression of Thrombospondin-1 in vascular smooth muscle cells, which has a mitogenic effect on the cells (Framson P, 1993). Thrombospondin-1 has also been implicated in the development of various pathophysiological conditions associated with the proliferation of smooth muscle cells (Schellings MW, 2004).

In contrast, cyclic GMP, produced in response to nitric oxide and natriuretic peptides by sGC, is a key regulator of vascular smooth muscle cell contractility, growth, and differentiation, and is implicated in opposing the pathophysiology of hypertension, cardiac hypertrophy, atherosclerosis, and vascular injury/restenosis (Pilz RB, 2003). The decrease in sGC α-1 expression by FCS reiterates its proliferative effect and provides a possible mechanism of this down-regulation. Thus FCS, down-regulates sGC α-1, which in turn lowers the cGMP content in the cellular environment which leads to the proliferation of smooth muscle cells. Indeed, expression of both sGC subunits (α-1 and β-1) was found decreased in aortic tissue of aged SHR as compared to aged WKY rats (Klöss, Bouloumie, Mülsch 2000).

5 Downregulation of sGC α-1 by serum is mediated by NFY and Sp1

In an effort to characterise the molecular mechanism of the serum-induced down-regulation of sGC, we studied the effect of serum on the activity of the transcription factors Sp1 and NFY, the main transcription factors identified in the constitutive expression of sGC α-1. Cell lysates from both control and FCS treated RLF-6 cells were used in the EMSA with Sp1 and NFY cognate DNA oligos. These studies revealed that in the presence of FCS, NFY binding decreased, whereas Sp1 binding increased (fig. 25), in comparison to serum-starved cells. This observation corroborated our previous findings that these factors regulate the expression of sGC α-1 in an opposite manner.

NFY and Sp1 are ubiquitous transcription factors involved in the constitutive and inducible expression of a lot of mammalian genes (Mantovani R., 1999);(Suske G., 1999). Sp1 has been found in the promoters of mouse sGC α-1 and human NPR-B gene (Garg R, 2002);(Rahmutula D, 2004). NF-Y plays an important role in the human β-1 sGC promoter regulation (Sharina IG, 2003). NFY- and Sp1-cognate sequences have been found in the mouse and human NPR-A promoter as well as in the rat NPR-A promoter where NFY and Sp1 act synergistically to

reconstitute NPR-A promoter activity (Liang F, 2001). A direct physical association between NFY and Sp1 has been demonstrated both in vitro and in vivo.

In case of rat sGC α–1 promoter where the binding sites for NFY and Sp1 overlap, we propose that under normal conditions, NFY is the dominat factor regulating promotor activity. However, the presence of FCS causes a shift in transcription factor activity, enabling Sp1 to bind to the same site and exert its suppressing effect. This was evident from the tests performed with the Alpha200 construct in the presence of Sp1 decoy and mithramycin A. The pathway leading to this shift caused by FCS remains to be elucidated. Also the involvement of other transcription factors besides NFY and Sp1 remains unknown.

6 Activation of AP-1 is responsible for the HuR mediated downregulation of sGC

The 3'-untranslated regions (UTR) of the sGC α-1 and β-1 mRNA bear AU-rich elements (ARE), which are targeted by *trans*-acting factors for regulation of mRNA stability. One of these factors is the *elav*-like ubiquitous 34-kDa protein HuR. Recently, HuR and other ARE-binding proteins were also found to regulate translation of target mRNAs, by recruitment to mammalian stress granules formed in response to metabolic stress (Kedersha N, 2002). HuR is down regulated in rat vascular smooth muscle cells (Kloss S, 2003) and mesangial cells (Akool el-S, 2003) by cGMP-eliciting agonists.

Forskolin, a cAMP-eliciting direct activator of adenylyl cyclase, and PKA-activating cAMP analogs were found to decrease the expression of both HuR and sGC in isolated rat aorta, at the protein and mRNA levels in a time-dependent fashion (Kloss S, 2004). In the absence of cAMP-eliciting conditions, a markedly slower decrease in HuR and sGC expression in rat aortic tissue was observed. The interaction of endogenous HuR with ARE-containing oligoribonucleotides

from the 3'-UTR of sGC α-1 and β-1 (EMSA) was strongly decreased in protein extracts from forskolin-exposed aorta, and this effect was blocked by Rp-cAMPS, an PKA-inhibitory cAMP analog. A decreased expression of HuR at the protein and mRNA levels was the most likely explanation for the reduced HuR sGC mRNA interaction. As a consequence of decreased HuR expression and mRNA binding activity, the stability of sGC α-1 and β-1 mRNA in in vitro degradation assays was considerably reduced by native protein extracted from forskolin-exposed aortic tissue, compared with protein from control aorta. These findings clearly show that HuR expression and activity is decreased by the cAMP/PKA signaling pathway (Kloss S, 2004). This mechanism probably accounts for decreased expression of sGC subunits, because HuR-dependent protection of sGC mRNA is lost. We cannot exclude the possibility that additional mechanisms could contribute to the cAMP-elicited decrease of sGC mRNA stability [e.g., induction of mRNA-destabilizing factors such as AUF 1, but HuR seems to play a pre-eminent role in sGC mRNA stability regulation.

It was also observed that forskolin induced a very rapid increase in Fos and activated p-Fos that was accompanied by a transient activation of AP-1, the heterodimeric transcription factor constituted by Fos and Jun. A causal relationship between AP-1 activation and HuR depression was established by an AP-1 decoy approach. Competition of an exogenously provided AP-1 cognate dsDNA oligonucleotide with endogenous AP-1 sites inhibited forskolin-induced depression of HuR. However, the complete sequence of signaling events leading to HuR depression by AP-1 activation remains to be elucidated. The mouse HuR promotor bears several AP1 and a conserved CREB site(s) (King PH, 2000), which could also mediate a direct silencing effect of cAMP on HuR promotor activity. It is noteworthy that quite similar signaling seems to account for NO/cGMP-induced down-regulation of HuR; it had been observed that the sGC activators YC-1 and sodium nitroprusside rapidly and transiently increased Fos and activated AP-1 in RASMC (S. Kloess, A. Muelsch, unpublished

observations), in accordance with the present paradigm of cGMP-dependent gene expression.

7 HuR interacts with the protein SSAT

In an effort to understand the intracellular interactions that regulate the activity of HuR in stabilizing ARE containing mRNA, several protein ligands to HuR have been identified and characterized (Brennan CM, 2000). They are SETa:b (Matsumoto K, 1993);(von Lindern M, 1992), pp32 (Malek SN, 1990) and acidic protein rich in leucine (APRIL) (Mencinger M, 1998). Deletion experiments suggested that the acidic tail of at least pp32 (and possibly these other HuR ligands) is required for their interaction with a region spanning the hinge region and third RRM of HuR (Brennan CM, 2000). SSAT (Spermidine/spermine N1-acetyltransferase) here has been found to interact with HuR through its hinge region. The hinge region of HuR also harbours its HNS (HuR Nucleocytoplasmic Shuttle) sequence (Fan XC, 1998b). It is yet to be investigated whether HuR ligands, including SSAT, increase the affinity of HuR for its target mRNAs or do they regulate HuR export, thereby modulating its ability to stabilize ARE-containing mRNAs.

We found here that the hinge region of HuR interacts with the catabolic enzyme SSAT. The oxidative catabolism of the higher order polyamines, spermidine and spermine, is accomplished by the concerted action of two different enzymes, SSAT and polyamine oxidase (PAO) (Casero RA Jr, 1993). Cytosolic SSAT N^1 acetylates both spermidine and spermine, which then serve as substrates for peroxisomal PAO. Because PAO strongly prefers acetylated polyamines to unmodified polyamines as its substrates, SSAT is generally considered the rate-controlling enzyme in the back conversion of the higher order polyamines, spermidine and spermine, to the lower order polyamine, putrescine. Thus far, spermine and spermidine are the only physiological substrates that have been

identified for SSAT (Chen C, 2004). Therefore, it is tempting to speculate that SSAT affects HuR functions by local effects on polyamines.

A direct effect of these proteins on each other's biological activity was not visible as tested through the SSAT activity assay and HuR gel shift. HuR failed to affect the enzymatic activity of SSAT and SSAT-GST did not have any effect on the gel shift performed by HuR on the 3' UTR of sGC α-1. However more direct/indirect assays need to be performed to unearth the functional rationale of this interaction. Indeed, previous studies have implicated polyamines in the regulation of cell cycle; Cyclin A expression is lowered in cells treated with polyamine biosynthesis inhibitors (Oredsson SM., 2003). HuR plays a critical role in cell proliferation, at least in part by mediating cell cycle-dependent stabilization of mRNAs encoding cyclins A and B1 (Wang W, 2000). Depletion of polyamines by treatment with DFMO, a drug that blocks the enzyme ornithine decarboxylase responsible for conversion of ornithine to putrescine, has been shown to alter the expression of cyclin A during cell cycle (Oredsson SM., 2003). NO inhibits VSMC proliferation by specifically changing the expression and activity of cell cycle regulatory proteins. Adenoviral overexpression of endothelial NOS represents a cytostatic strategy for gene therapy of vascular disease (Tanner FC, 2000). Thus, it is conceivable that SSAT-mediated modulation of local polyamine concentrations enhances/reduces HuR activity and sGC expression to affect cell proliferation. Since SSAT interacts with HuR through its hinge region, which has the nucleocytoplasmic shuttling sequence, it is possible that it affects the mRNA-transporting function of HuR. However, an equally plausible hypothesis is that SSAT is involved in acetylating other unknown substrates whose acetylation affects their role in the cell cycle. However, it still needs to be investigated how the HuR-SSAT interaction affects sGC expression and mRNA stability as well as the effect of these in cell proliferation.

V Summary

Soluble guanylyl cyclase (sGC) is a cytosolic enzyme producing the intracellular messenger cyclic guanosine monophosphate (cGMP) on activation with nitric oxide (NO) which leads to the activation of GMP dependent protein kinases and to vasodilation. NO signaling may be affected by altered expression of sGC subunits, as has been shown in different pathological and physiological conditions and developmental stages. The molecular mechanisms underlying altered sGC expression in these and other conditions have not yet been revealed.

Gene expression can also be regulated at the level of mRNA through alterations in translational efficiency and in mRNA stability. HuR (Human R) is a ubiquitously expressed member of the embryonic lethal abnormal vision (ELAV) family of RNA-binding proteins. Among other RNAs, there has been recent evidence that the expression of sGC is subject to post-transcriptional regulation by HuR. It has been shown that chronic hypertension induces changes in HuR expression and activity, which account for decreased sGC expression and activity in the aorta of hypertensive rats.

This thesis should study was performed in an effort to provide some insight to the transcriptional and post-transcriptional regulation of sGC expression in a mammal, the rat.

We investigated rat sGC α-1 transcriptional regulation in rat lung fibroblast (RLF-6) cells. The 3000bp 5' upstream region of the α-1 sGC gene was isolated and analyzed for promoter activity by using luciferase reporter constructs- Alpha3000 (with -2794 bp), Alpha1100 (-1092 bp), Alpha350 (-346 bp) and Alpha200 (-200 bp). The promoter activity was the highest in the 200bp construct (about 6-fold higher than Alpha3000) suggesting that this fragment contains all

the crucial elements necessary to support basal transcription of the α-1 sGC gene.

Analysis of the 200 bp of the 5' UTR of the α−1 gene was performed using the MATINSPECTOR V2.2 software for putative transcription factors. The constructs containing the deleted sites for NFY and Sp1 showed a significant decrease in constitutive promoter activity by almost 80% and 60% respectively, implying that these transcription factors are crucial elements in the basal expression of the of sGC α−1 subunit. Treatment of RLF-6 cells with genistein 50 μM and mithramycinA 100 nM, known to inhibit the NFY and Sp1 binding to DNA respectively, reflected the same effects. Furthermore the cGMP content of the cells was significantly reduced by both inhibitors, almost completely by genistein, and by about 40 % by mithramycinA.

Electrophoretic mobility-shift assay (EMSA) clearly showed the formation of multiple complexes with the biotinylated ODN (decoy oligodeoxynucleotide) probes for NFY and Sp1 when incubated with RLF-6 nuclear extract. A "supershift" observed in the presence of antibodies to the individual transcription factors confirmed that these factors were present in the shifted band, indeed.

NFY and Sp1 are instrumental in several physiological and pathophysiological effects mediated by several growth factors in smooth muscle cells. Thus the regulation of the promoter, in response to serum, was also analysed. 10% foetal calf serum led to decreased α-1 sGC level as shown by western blots performed with rat aorta. Decreased sGC α-1 mRNA expression was observed in RLF-6 cells and cultured rat aortic smooth muscle cells incubated with FCS for 24 hours. This decrease was reflected in the promoter activity in RLF-6 cells using both Alpha3000 and Alpha200 constructs confirming that the regulation took place at promoter level.

EMSA performed with nuclear extracts from FCS treated RLF-6 cells led to diminished binding to NFY, but to an enhanced binding to Sp1 site. We concluded that the factors Sp1 and NFY (the sites overlapping) compete for binding, and in the presence of FCS, it is Sp1 that binds stronger, and hence results in diminishing promoter activity.

In order to delineate the post-transcriptional regulation of sGC α-1 subunit, studies were performed to demonstrate the regulation of expression of the mRNA stabilizing protein HuR. It has been observed that exposure of isolated rat aortic segments to the activator of adenylyl cyclase, forskolin, strongly reduced sGC $\alpha-1/\beta-1$ and HuR protein and mRNA expression in a time-dependent and actinomycin D-sensitive fashion. Transcription factor decoy approach proved that the cAMP-induced down-regulation of HuR is mediated by the activation of AP-1.

It has been established that HuR stabilises the sGC $\alpha-1$ and β-1 mRNA. However the pathway underlying this regulation remains unknown. In order to identify the mechanism of this regulation, we looked for HuR interacting proteins employing the yeast two hybrid assay. The enzyme of the polyamine catabolic pathway spermidine/spermine N1-acetyltransferase (SSAT) was found to interact with the hinge region of HuR. This interaction was confirmed by performing immunoprecipitation and GST-pulldown experiments. A direct effect of these proteins on each other's biological activity was not visible as tested through the SSAT activity assay and HuR gel shift. It might be possible that SSAT-mediated modulation of local polyamine concentrations enhances/reduces HuR activity and sGC expression to affect cell proliferation.

In summary, this study represents an analysis of the rat sGC α-1 promoter regulation in rat fibroblast cells and identifies NFY and Sp1 as important factors in sGC α-1 expression. It also gives first evidence of sGC regulation at the transcriptional level in response to an external stimulus, and proposes the possible mechanism. It also identifies SSAT as a HuR interacting protein. These

might have implications in the various pathophysiological conditions where sGC plays an important role.

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VII Appendix

1 Abbreviations

AC	Adenylyl cyclase
Act. D	Actinomycin D
ADP	Adenosine diphosphate
Amp	Ampicillin
ANF	Atrial Natriuretic Factor
AP-1	Activator protein 1
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
cAMP	cyclic Adenosine monophosphate
cGMP	cyclic Guanosine monophosphate
CRE	cAMP responsive element
kDa	kilo Dalton
dATP	Deoxy-Adenosine triphosphate
dCTP	Desoxy-Cytidine triphosphate
DEPC	Diethylpyrocarbonate
dGTP	Desoxy-Guanosine triphosphate
DNA	Deoxyribo-nucleic acid
dNTP	Deoxy-nucleotide triphosphate
DTT	Dithiothreitol
dTTP	Deoxy-Thymidine triphosphate
EDRF	“endothelium derived relaxing factor“
EDTA	Ethylene diamine tetraacetate
eNOS	endothelial NO-Synthase
GTP	Guanosine-5‘-triphosphate
h	hour
HuR	Human Antigen-R

iNOS	inducible NO-Synthase
IPTG	Isopropylthiogalactoside
kb	Kilobase
kDa	Kilodalton
L-NAG	N-Nitro-L-Arginine
min	Minute
mg	Milligramm
µM	Micromole per Liter
µl	Microliter
ml	Milliliter
mM	Millimol per Liter
mRNA	messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
nmol	Nanomol
NS2028	4H-8-bromo-1,2,4-oxadiazolo(3,4-d)benz(b)(1,4)oxazin-1-on
NTG	Nitroglycerine
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered-saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pGC	Particulate guanylyl cyclase
PMSF	Phenylmethylsulfonylfluoride
RNA	Ribonucleic acid
RP-8-Bromo-PET-cGMPS	ß-Phenyl-1,N^2-ethene-bromoguanosine 3'-5'cyclic monophosphorothioate
rRNA	ribosomal Ribonucleic acid

RT-PCR	Reverse Transcriptase-Polymerase-reaction
sec	Seconds
SDS	Sodiumdodecylsulfate
SHR	“spontaneously hypertensive rats“
SMC	Smooth muscle cells
SNP	Sodiumnitroprusside
SSAT	Spermidine/spermine N1-acetyl-transferase
TBE	Tris-Borate-EDTA
TEMED	Tetramethylethylendiamine
Tris	Tris-(Hydroxymethyl)-aminomethane
tRNA	transfer Ribonucleic acid
U	Unit (s)
V	Volts
Vol	Volumes
WKY	Wistar-Kyoto-Ratten
YC-1	3-(5’-hydroxymethyl-2’-furyl)-1-benzyl indazol

2 Zusammenfassung

Die lösliche Guanylatzyklase (soluble guanylyl cyclase, sGC) ist ein Schlüsselenzym der NO/cGMP Signaltransduktion im kardiovaskulären System. Nach Aktivierung der sGC durch Stickstoffmonoxid (NO) kommt es in glatten Muskelzellen zur Produktion von zyklischem 3',5'-Guanosinmonophosphat (cGMP), das als intrazelluläres Signaltransduktionsmolekül die Aktivierung von cGMP-abhängigen Proteinkinasen vermittelt und zur Vasodilatation führt.

Die NO/cGMP-Signaltransduktion kann auch auf der Ebene der Gen-Expression beeinflusst werden. So konnte in verschiedenen physiologischen bzw. pathophysiologischen Modellen gezeigt werden, dass die unterschiedliche Expression von sGC-Untereinheiten die NO/cGMP-Signaltransduktion beeinflusst.

Die molekularen Mechanismen, die auf der unterschiedlichen Expression der sGC-Untereinheiten basieren, wurden bisher noch nicht ausreichend aufgeklärt.

Die Gen-Expression kann auf mRNA-Ebene durch Veränderung der Translation und der mRNA- (messenger ribonucleic acid, Boten-Ribonnukleinsäure) Stabilität reguliert werden. HuR (Human R) ist ist ein ubiquitär vorkommendes Mitglied der embryonic lethal abnormal vision (ELAV-) Familie der RNA-bindenden Proteine. Neben der Regulierung anderer Gene gibt es Hinweise darauf, dass auch die Expression der sGC einer post-translationalen Regulation durch HuR unterliegt. So wurde gezeigt, dass chronischer Bluthochdruck Veränderungen in der HuR-Expression und -Aktivität verursacht, was zu einer verminderten sGC-Expression und -Aktivität in der Aorta von hypertensiven Ratten führt.

Diese Arbeit soll einen Einblick in die transkriptionale und post-transkriptionale Regulation der sGC-Expression im Rattenmodell liefern.

Im Rahmen dieser Arbeit wurde die translationale Regulation der α-1 sGC-Untereinheit der Ratte in Rattenlungen-Fibroblastenzellen (rat lung fibroblast cells, RLA-6) untersucht.
Es wurden die 3000 bp der 5´ upstream Region des Gens der α-1 sGC-Untereinheit isoliert und mit Hilfe von Luziferase-Reporter-Konstrukten - Alpha3000 (mit -2794 bp), Alpha1100 (-1092 bp), Alpha350 (-346 bp) und Alpha200 (-200 bp) - auf Promotor-Aktivität untersucht. Dabei war die Promotor-Aktivität des 200 bp Konstrukts am höchsten (ca. 6-fach im Vergleich zu Alpha3000). Dies deutete daraufhin, dass dieses Fragment entscheidende Elemente enthält, die für die basale Transkription des α-1 sGC Gens nötig sind.

Um nach vermeintlichen Transkriptionsfaktoren zu suchen, wurde die Analyse der 200 bp am 5´ UTR des α-1 Gens mit der MATINSPECTOR V2.2 Software durchgeführt. Die Konstrukte mit den entfernten Stellen für NFY und Sp1 zeigten eine signifikante Verminderung in der konstitutiven Promotor-Aktivität zu fast 80 und 60%. Dies deutete daraufhin, dass diese Transkriptionsfaktoren entscheidend sind für die basale Expression der α-1 sGC-Untereinheit. Die Behandlung der RLF-6 Zellen mit Genistein (50 µM) und Mithramycin A (100 nM), die die Anbindung von NFY und Sp1 an die DNA inhibieren, führte ebenfalls zu einer Reduktion der Promotor-Aktivität. Außerdem war der Gehalt von cGMP in den Zellen durch die beiden Inhibitoren signifikant reduziert - mit Genistein wurde eine fast komplette Inhibition erreicht und mit Mithramycin A eine Reduktion um ca. 40%.

Der EMSA (electrophoretic mobility-shift assay) zeigte deutlich die Bildung von multiplen Komplexen mit biotinylierten ODN (decoy oligodeoxynucleotide) Proben für NFY und Sp1, wenn diese mit RLF-6 Zellkern-Extrakt inkubiert wurden. In einem „supershift“ konnten die einzelnen Transkriptionsfaktoren mit Antikörpern nachgewiesen werden.

NFY und Sp1 sind an zahlreichen physiologischen und pathophysiologischen Prozessen in glatten Muskelzellen beteiligt, die durch Wachstumsfaktoren gesteuert werden. Aus diesem Grund wurde die Regulation des Promotors in Gegenwart von Serum analysiert.

Mit Western-Blot Analysen konnte gezeigt werden, dass 10 % fötales Kälber-Serum (foetal calf serum, FCS) zu einer verminderten Expression von α-1 sGC in Rattenaortenringen führte. Eine verminderte sGC α-1 mRNA-Expression wurde in RLF-6 Zellen und in kultivierten glatten Muskelzellen der Rattenaorta, die mit FCS über 24 Stunden inkubiert wurden, demonstriert. Diese Verminderung ging einher mit einer verminderten Promotor-Aktivität der Konstrukte Alpha3000 und Alpha200 in RLF-6 Zellen, was eine Regulation auf Promotor-Ebene aufzeigte.

EMSA Studien mit Zellkern-Extrakt aus FCS-vorbehandelten RLF-6 Zellen zeigten eine verminderte NFY-, aber eine gesteigerte Sp1-Bindung. Daraus wurde gefolgert, dass die Faktoren Sp1 und NFY um eine Bindungsstelle am Promotor konkurrieren und dass die Gegenwart von FCS zu einer verstärkten Sp1-Bindung und damit zu einer verminderten Promotor-Aktivität führt.

Um die post-transkriptionale Regulation der α-1 sGC-Untereinheit zu demonstrieren, wurden Versuche zur Regulation der Expression des mRNA-stablisierenden Proteins HuR durchgeführt. Es konnte gezeigt werden, dass die Inkubation von isolierten Rattengefäßringen mit Forskolin, einem Aktivator der Adenylatzyklase, die sGC α-1/β-1 und die HuR Protein- und mRNA-Expression zeitabhängig und Actinomycin D-sensitiv reduziert. Der Transkriptionsfaktor-Abbau bewies, dass die cAMP-induzierte Runterregulation von HuR durch die Aktivierung von AP-1 vermittelt wird.

Es konnte festgestellt werden, dass HuR die α-1 und β-1 mRNA der sGC stabilisiert. Aber die Regulation dieser Signalwege war bisher unbekannt. Um den Mechanismus der Regulation zu identifizieren, haben wir nach HuR-interagierenden Proteinen durch Anwendung des Yeast-two-hybrid assays gesucht. Die Enzyme des Polyamin-katabolischen Signalwegs Spermidin/ Spermin N1-Acetyltransferase (SSAT) wurden als eine hinge Region von HuR entdeckt. Diese Interaktion wurde bestätigt durch Immunopräzipitation und GST-pulldown Experimente. Einen direkten gegenseitigen Effekt auf die Aktivität dieser Proteine war nicht sichtbar, was durch SSAT activity assays und HuR gel shifts getested wurde. Es könnte möglich sein, dass die SSAT-vermittelte Modulation der lokalen Polyamin-Konzentration die HuR-Aktivität und die sGC-Expression fördert bzw. vermindert bei der Zellproliferation.

Diese Arbeit zeigt eine Analyse der Promotor-Regulation der α-1 sGC-Untereinheit der Ratte in Fibroblastenzellen der Ratte und identifiziert NFY und Sp1 als wichtige Faktoren in der α-1 sGC-Expression.

Die sGC-Regulation auf transkriptionaler Ebene unter Beteiligung der Transkriptionsfaktoren NFY und Sp1, ausgelöst durch Serum als externen Stimulus, weist auf einen möglichen Mechanismus hin. Außerdem wurde SSAT als HuR-Interaktionsprotein identifiziert. Die in dieser Arbeit dargestellte Regulation der sGC-Expression könnte Einfluss haben auf verschiedene pathophysiologische Zustände.

3 List of Figures

4 List of Tables

5 Curriculum Vitae

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M.Sc. - "Investigations into autoimmune anti-lymphocyte antibody responses."

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A liquid synchronized-growth culture assay for the identification of true positive and negative yeast three-hybrid transformants.

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Rashi Srivastava and SK Lal

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Deutschen Gesellschaft für Kardiologie - Herz- und Kreislaufforschung: 24 - 26 April 2003, Mannheim, Germany.

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