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Identification and characterization of SM-20, a novel growth factor-responsive gene in vascular smooth muscle

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City University of New York, 1994

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Identification and Characterization of SM-20, a Novel Growth Factor-responsive Gene in Vascular Smooth Muscle

by

Stephen D. Wax

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

Identification and Characterization of SM-20, a Novel Growth-related Gene in Vascular Smooth Muscle

by

Stephen D. Wax

Adviser: Associate Professor Mark B. Taubman

Vascular smooth muscle cell (VSMC) growth contributes to the pathogenesis of atherosclerosis, hypertension, restenosis following balloon angioplasty, and the neovascularization of solid tumors. To identify genes whose expression levels are regulated during VSMC growth, we utilized differential screening of a rat VSMC cDNA library. A novel gene, SM-20, was identified whose corresponding mRNA is approximately 3 kb. SM-20 mRNA was expressed in quiescent VSMC (baseline), and was rapidly increased 3-4 fold after one hour of treatment with the proliferative agonists serum and platelet-derived growth factor (PDGF), the hypertrophic agent angiotensin II, and the vasodilatory agents forskolin and isoproterenol. mRNA levels rapidly returned to baseline by 3 hours. The induction of SM-20 by serum was not dependent on new protein synthesis, indicating that SM-20 is part of the immediate-early response to growth factor stimulation. Unlike other immediate-early genes studied in VSMC, SM-20 mRNA was not detected in fibroblast culture. Examination of SM-20 mRNA levels in adult rat tissues demonstrated high levels of expression in cardiac, skeletal, and smooth muscle-containing tissues, as well as the brain. Antibodies were raised against a 230 amino acid portion of

the putative SM-20 peptide sequence expressed in E. coli. Western blot analysis of VSMC lysates revealed one major protein species that migrated with an apparent molecular weight that is consistent with the predicted value of 39.7 kD. Immunohistochemistry of the adult rat aorta revealed that SM-20 protein is present in medial smooth muscle cells, and was markedly elevated in the smooth muscle cells comprising the neointimal plaque formed after balloon injury. SM-20 protein was not detected in the adventitial fibroblasts or in the endothelium. Immunostaining of human atherosclerotic coronary arteries demonstrated a similar pattern of SM-20 expression as seen in the rat model. Immunostaining of C2C12 myoblast cultures revealed that SM-20 is markedly induced following myogenic differentiation. SM-20 is a novel tissue-specific gene that may play a critical role in VSMC growth as well as myogenic differentiation.

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Chapter 1: Introduction

Atherosclerosis is the leading cause of death in the United States. Central to the progression of this disorder is the proliferation of vascular smooth muscle cells (VSMC) within the vessel wall (Schwartz et al, 1986a). VSMC proliferation is involved in the neovascularization of solid tumors. VSMC proliferation and hypertrophy (increase in cell size but not cell number) are also seen in the vessels of patients with hypertension. The focus of this thesis is to identify a novel gene that encodes a potential regulator of VSMC growth.

Pathology of the vessel wall

Adult mammalian arteries are organized in concentric rings (Fawcett, 1986). The inner ring, referred to as the tunica intima, consists of a single layer of endothelium which lies on a basement membrane. The endothelial cells function as a selective permeability barrier and a source of cytokines and other regulatory molecules. The tunica media is comprised of several layers of VSMC separated by elastic laminae. These VSMC are in a "contractile state", in which most of the cytoplasm (approximately 70%) consists of myofilament bundles composed of actin and myosin (Campbell et al, 1988). These contractile cells respond to neural and humoral inputs to regulate the tension in the arterial wall, and thus to regulate blood pressure. The contractile VSMC contain small amounts of synthetic organelles, such as rough endoplasmic reticulum, Golgi, and free ribosomes. These cells are thought to produce the connective tissue within the arterial

media. The outermost layer of the vessel, the tunica adventitia, consists of fibroblasts in a dense connective tissue matrix.

As its name suggests, atherosclerosis involves the progressive development of a fibrous-fatty plaque, referred to as the neointima, in the intima of the vessel (Ross, 1993). The formation and expansion of atherosclerotic plaque into the lumen can restrict blood flow, causing a shortage of oxygen to the downstream tissues. In addition, atherosclerotic plaques are often sites of clot formation (thrombosis), which can completely occlude the artery and cause infarction of the downstream tissues (Fuster et al., 1992). This process can occur throughout the arterial system. However, the coronary arteries, which supply the myocardium, and the carotid arteries, which supply the brain, appear to be particularly susceptible. These organs have high levels of metabolic activity, and thus are especially sensitive to compromised blood flow.

VSMC are the predominant cell type in the atherosclerotic plaque (Dartsch et al, 1989). These VSMC are in a "synthetic state", morphologically characterized by few cytoplasmic myofilaments and abundant synthetic organelles (Campbell et al, 1988). These cells synthesize extracellular matrix, which may contribute to the size, rigidity, and thrombogenic properties of the plaque. The VSMC are thus at the center of the atherosclerotic process. Other cell types found in less abundance include macrophages, lymphocytes, and fibroblasts (Ross, 1993).

Origin of the neointimal VSMC

The shift of VSMC between the synthetic and contractile phenotypes resembles that seen during development of the vasculature. The vasculature is formed when endothelial precursors, hemangioblasts, undergo differentiation and morphogenesis to form tube-like structures which invaginate into the parenchyma of the target organs. Locally derived mesenchymal cells condense around the endothelium, and undergo determination and partial differentiation into smooth muscle (as assessed by expression of markers such as smooth muscle α-actin) (Schwartz et al, 1990b). Neonatal ("pup") VSMC possess a synthetic phenotype and proliferate until the vessel reaches adult size. The VSMC then acquire the adult contractile phenotype. VSMC of the atherosclerotic plaque closely resemble the neonatal VSMC in morphology. The shared phenotype between the atherosclerotic and neonatal VSMC has been referred to as "pup-intimal" (Majesky et al, 1992).

The morphologic relationship between the neonatal and neointimal VSMC is paralleled by patterns of gene expression. Osteopontin, tropoelastin, α1 procollagen, PDGF-B chain, β-actin, and non-muscle myosin heavy chain ("Semb") are expressed in neonatal VSMC, repressed in the adult, and are then re-expressed in neointimal VSMC (Giachelli et al., 1991; Majesky et al., 1992; Kuro-o et al, 1991). It has been thought that either the neointimal cells of the plaque result from the "de-differentiation" of contractile cells, or are derived from undifferentiated smooth muscle present in the vessel wall.

The transformation of VSMC from the contractile to the synthetic phenotype has been studied *in vitro*. When smooth muscle of the tunica media are first placed into culture, the cells retain a contractile, nonproliferative phenotype. In the presence of platelet-derived serum, subconfluent VSMC undergo "phenotypic modulation" to a synthetic morphology and will begin to proliferate (Campbell et al, 1988). Re-differentiation of some VSMC to the contractile phenotype can occur if the cells reach confluence within 5 cell doublings and 15 days in culture. If the cells are passaged beyond these parameters, then the synthetic phenotype becomes irreversible.

There is some evidence to suggest that a subpopulation of VSMC in the vessel wall may be predisposed to proliferate under pathologic conditions and contribute to plaque formation (Schwartz et al, 1990b). This is analogous to skeletal muscle tissue, which contains pools of undifferentiated stem cells, referred to as satellite cells. Unlike the differentiated, striated muscle fibers, these satellite cells possess a synthetic phenotype, and can replicate and repair damaged tissue. In newborn humans, there are synthetic-type smooth muscle cells scattered within the tunica intima (Schwartz et al, 1990b). In addition, there are cells within the arterial media which do not stain for differentiation markers, such as desmin (Kocher et al, 1985). Within all cultures of rat aortic smooth muscle cells, there is heterogeneity in the requirement for platelet-derived growth factors for proliferation (Schwartz et al, 1990a).

Proliferation of VSMC in human atherogenesis

The importance of VSMC proliferation to human plaque formation was first demonstrated by Benditt and Benditt (1973). Analysis of neointimal lesions from women heterozygous for glucose 6-phosphate dehydrogenase (G-6-PD) demonstrated that, in over 80% of the samples, one isozyme accounted for greater than 95% of the total enzyme in the plaque. In contrast, samples of the underlying media revealed a mixture of isozyme-specific VSMC. This suggested that the VSMC of the plaque were generated by a focal, monoclonal proliferation of medial VSMC.

Gordon et al (1990) demonstrated the presence of proliferating cell nuclear antigen (PCNA) in smooth muscle cells of human coronary plaques. This protein is a processivity factor for DNA polymerase δ, and is expressed during S-phase. Detection of PCNA thus indicated that these cells were proliferating.

The above findings are in agreement with the conclusions drawn from many experimental animal models of atherogenesis, in which the role of VSMC proliferation has been firmly established (Ross, 1993).

Migration of medial VSMC

The contribution of migrating VSMC to the neointima has been most clearly demonstrated in experimental animal models. Balloon injury to a rat aorta leads to the accumulation of synthetic VSMC in the intima. VSMC are not found in the intima of normal rat arteries, and thus it has been concluded that migration of medial VSMC must have occurred (Clowes and Schwartz, 1985). The migration of VSMC

out of the media may release these cells from growth inhibitory influences, and thus contribute to the subsequent proliferation of VSMC in the intima.

Regulators of VSMC neointimal accumulation

The molecular regulators of neointimal formation have been elucidated in large part by studying the cellular response to arterial injury. In particular, balloon angioplasty of an intact artery (performed by repeated dragging of a balloon-tipped catheter across the vessel lumen) leads to the accumulation of a neointima rich in synthetic smooth muscle cells (Schwartz et al, 1975). In the rat carotid injury model, basic fibroblast growth factor (bFGF) has been implicated as crucial to the initiation of VSMC proliferation. Infusion of antibodies to bFGF at the time of balloon injury inhibited the proliferative response of the VSMC (Lindner and Reidy, 1991a); likewise, infusion of bFGF enhanced VSMC proliferation (Lindner et al, 1991b). In this model, the source of bFGF was likely to be intracellular stores released from dead or dying cells of the vessel wall, or extracellular matrix molecules which normally sequester the bFGF peptides.

PDGF appears to be the primary mediator of VSMC migration into the intima. In the rat arterial injury model, anti-PDGF (Ferns et al, 1991) or anti-platelet (Fingerle et al, 1989) antibodies inhibited the accumulation of VSMC in the intima, but did not markedly affect the proliferative response. Infusion of PDGF-BB increased intimal thickening 20 fold due in large part to the increased migration of

medial VSMC; there was only a 2-3 fold increase in medial VSMC proliferation (Jawien et al, 1992).

Following balloon injury, the endothelium is completely denuded and a "carpet" of platelets lines the vessel (Schwartz et al, 1975). The platelets release growth factors, cytokines, and matrix-degrading enzymes. This release can persist for several days. Platelet deposition appears to be markedly diminished after 48-72 hrs (Reidy et al, 1986). However, proliferation of VSMC continues for several weeks (Clowes and Schwartz, 1985). This suggests the presence of an autocrine/ paracrine growth mechanism within the vessel wall. In VSMC culture, bFGF increased the number of PDGF α-receptors (Schollmann et. al, 1992). Using the balloon injury model, Majesky and colleagues (1990) demonstrated that the PDGF A-chain and β-receptor were upregulated in the neointimal VSMC.

The importance of growth inhibitors in the wall of an intact artery has also been shown using the balloon injury model. It is likely that VSMC are maintained in a quiescent state *in vivo* by a combination of the absence of growth stimulants and the presence of growth inhibitors. One such class of inhibitors is the glycosaminoglycans, in particular the heparan sulfates which surround the VSMC. Heparin was found to inhibit VSMC proliferation when infused into the vessel at the time of balloon injury (Clowes and Karnowsky, 1977; Majesky et al, 1987). *In vitro* studies have determined that heparin can directly block VSMC growth. These matrix molecules can also exert an indirect negative effect on growth *in vivo* by sequestering growth factors, particularly acidic FGF and bFGF.

TGF-β is also present in the intact vessel wall (Majesky et al., 1991), and may contribute to VSMC growth inhibition (Owens et al, 1988). Interestingly, TGF-β is markedly induced in the medial VSMC 6 hrs after balloon injury, and remains elevated in the neointimal VSMC after 2 weeks (Majesky et al., 1991). Under certain conditions, TGF-β can stimulate the proliferation of VSMC *in vitro* (Battegay et al, 1990). Thus the induction of TGF-β after injury may actually promote VSMC growth.

A model for the pathogenesis of atherosclerosis

The above mentioned studies (and others) have led to a model for the progression of atherosclerosis as a "response to arterial injury" (Ross, 1993). Minor injuries occur to the vessel wall, particularly the endothelial cells, by agents such as oxidized LDL or the sheer stress of turbulent blood flow. This activates an inflammatory repair response. Monocytes are recruited to the site of injury, where they differentiate into macrophages. The uptake of lipid by these macrophages results in the formation of "foam cells" which form a layer beneath the endothelium referred to as a "fatty streak".

The formation of a fatty streak is an early finding in coronary arteries of 10-12 year old patients, and is also seen within one month of cholesterol feeding of primates (Stary 1983; Masuda and Ross, 1990a). Fatty streaks often occur at the same sites in the coronary vasculature, such as vessel branch points, in which the

advanced lesions of the adult are found. This indicates that the fatty streak is likely to be a precursor to advanced atherosclerotic lesions.

Activated macrophages may release endoglycosidases and other matrix degrading enzymes which can release bound bFGF, initiating VSMC growth. Macrophages also synthesize PDGF (Ross et al., 1990) which can cause migration of VSMC into the intima and continued proliferation of the VSMC (PDGF is a potent mitogen for VSMC in culture). The migration of VSMC leads to diffuse intimal thickening. The neointimal VSMC synthesize PDGF ligand as well as receptor, which could lead to autocrine and paracrine stimulation of VSMC growth and migration. These VSMC accumulate intracellular lipid. Interestingly, the M-CSF receptor is also expressed by neointimal VSMC (Inaba et al., 1992). Treatment of these cells cultured *in vitro* with M-CSF leads to increased uptake of acetylated-LDL, and ultimately to a foam cell-like appearance.

Concomitant with the development of a neointima is the disruption of the endothelial lining. This exposes the connective tissue, VSMC, and macrophages to the circulation (Masuda and Ross, 1990b). Platelets adhere to these thrombogenic surfaces releasing growth factors and cytokines, which contribute to the continued expansion of the atherosclerotic plaque. Lipids increasingly accumulate extracellularly and intracellularly. The production of extracellular matrix, especially type V collagen, by the VSMC is likely to be stimulated by elevated levels of TGF-β in atherosclerotic plaques (Lawrence et al, 1994). This plaque can ultimately become encapsulated by a fibrotic cap.

Molecular pathways controlling cell growth

As outlined above, the proliferation of vascular smooth muscle is central to the pathogenesis of atherosclerosis. An understanding of the cellular processes occurring during the transition of quiescent cells into the cell cycle has been derived from studies of cultured fibroblasts. Incubation of fibroblasts in serum-free medium diminished ^{3}H-thymidine incorporation to less than 5% of the level observed in serum-treated cells. These cells entered G0, a distinct physiologic state characterized by a smaller cell volume, decreased metabolic activity, and ribosomes present largely as monosomes, rather than polysomes (Pardee, 1989). To initiate proliferation, particular growth factors were required. For fibroblasts and smooth muscle cells, these growth factors included PDGF or bFGF, which are not normally present in plasma (Ross et al., 1974). After several hrs of treatment with growth factors, the fibroblasts reached a stage referred to as competence. Competent cells traversed the rest of the cell cycle in the presence of platelet-poor plasma (Pledger et al, 1977). IGF-1 was found to be the most potent growth-promoting "progression factor" in plasma (Stiles et al, 1979), whereas the essential amino acids were critical permissive factors (Pledger et al, 1978). These findings were also observed in smooth muscle (Clemmons, 1984). Interestingly, PDGF increased the synthesis of the IGF-1 receptor in fibroblasts (Clemmons et al, 1980), as well as the synthesis and secretion of IGF-1 ligand in smooth muscle (Delafontaine et al, 1991). This suggested a potential for autocrine and paracrine promotion of cell growth.

Cell cycle progression depends on extracellular signals through most of the G1 phase until approximately 2 hrs before the onset of DNA synthesis, referred to as "R". After this point, traverse of the mitotic checkpoints in the cell cycle requires intracellular signals that ensured the fidelity of the replication process (Pardee, 1989).

The intracellular molecular processes which occur following stimulation by competence factors is exemplified by the cellular response to PDGF stimulation. Detailed analyses of the PDGF receptor structure and function were largely done with the β-receptor. The primary sequence of the α and β receptors are highly homologous, however, and are thus likely to share many characteristics (Claesson-Walsh et al, 1989). The extracellular portion of each PDGF receptor consists of 5 immunoglobulin-like domains. This type of repeated structure occurs in a number of extracellular molecules which bind specific targets, including growth factor receptors, adhesion molecules, and, of course, immunoglobulins. A single transmembrane region and a "juxtamembrane" region is followed by the tyrosine kinase domain, which is interrupted by a "kinase insert" region.

The PDGF ligand is a dimer. Binding of each subunit to a single receptor induces a conformational change and cross-tyrosine phosphorylations of the intracytoplasmic regions of the two receptors. Specific phosphotyrosines of the activated receptor then bind signal transducing molecules via their SH2 domains (Fantl et al 1992). A cascade of activation of kinases and phosphatases is triggered, altering the activity of many regulatory molecules (Williams, 1989). These include phosphatidylinositol-3' (PI-3) kinase, phospholipase Cγ, ras-GTPase Activating Protein (GAP), raf-1, and src

and its related kinases. Activation of PLC results in the formation of diacylglycerol and IP3, leading to the activation of protein kinase C and calcium release from intracellular stores, respectively. The Na^+/H^+ antiporter is rapidly activated, leading to cytoplasmic alkalinization and increased intracellular Na^+. Prostaglandin synthesis is also increased by the sequential actions of phospholipase A and cyclooxygenase. These prostaglandins bind to G-protein linked receptors, leading to increases in intracellular cAMP concentrations. The combinatorial action of second messengers then regulates gene expression, which ultimately determines the growth state of the cell.

The transcription of new mRNA (Smith and Stiles, 1981) is an obligatory event in the transition of cells to competence. In searching for specific mRNA species that were regulated by PDGF stimulation of quiescent cells, the proto-oncogene c-*myc* was found to be rapidly induced. This was of particular significance because abnormal regulation of this gene product was linked to the development of the transformed phenotype. c-*myc* mRNA was induced even when de novo protein synthesis was blocked, indicating that the induction was a direct result of growth factor treatment and not just a consequence of cell growth (Kelly et al., 1983). Transcriptional activation of the protooncogenes c-*fos*, as well as c-*jun*, was also demonstrated (Greenberg and Ziff,1984; Ryder and Nathans, 1988). These studies suggested a mechanism by which gene activation by growth factors was linked to subsequent cellular proliferation.

Initial experiments pointed to a critical role in cell proliferation for protooncogenes. Overexpression of these genes enhanced

proliferation and inhibited withdrawal from the cell cycle (Sorrentino et al., 1986; Askew et al, 1991). Inhibition of these genes by antisense or antibody techniques prevented cellular proliferation and enhanced differentiation (Nishikura and Murray, 1987; Heikkila et al, 1987; Prochownik et al, 1988). The importance of these immediate early genes in VSMC was underscored by studies with c-*myc* and c-*myb*. Antisense oligonucleotides which blocked the synthesis of c-myc protein inhibited VSMC proliferation and migration *in vitro* (Biro et al, 1993) and decreased neointimal formation following rat carotid artery injury (Bennet et al, 1994). Antisense oligonucleotide inhibition of c-*myb* synthesis blocked VSMC proliferation *in vitro* and neointimal accumulation *in vivo* (Brown et al, 1992; Simons et al, 1992).

The control of cell growth, however, does not always parallel the regulation of the proto-oncogenes. In fibroblasts the activation of c-*myc* was neither sufficient nor necessary for mitogenesis (Coughlin et al, 1985), and in B cells c-*myc* activation was not sufficient for mitogenesis (Smeland et al, 1985). c-*myc*, c-*fos*, and c-*jun* were activated during cellular processes other than proliferation. These include the NGF-induced differentiation of PC12 cells (Greenberg et al, 1985), and the hypertrophic growth of post-mitotic cardiocytes (reviewed by Chien et al, 1991). In addition, c-*fos*, c-*jun* and their related family members induced the differentiation of myeloid cells (Lord et al, 1993).

Identification of novel growth-related genes

Williams and Penman (1975) used Rot curve analysis to estimate that logarithmically growing cells and quiescent cells each specifically express several hundred mRNA species. To search for novel genes that are regulated by growth factors, several groups utilized differential screening. This technique involves isolating cDNA clones which correspond to mRNA species whose abundance differs between two populations of cells (i.e. growth factor treated and untreated). A cDNA library is plated, and duplicate nitrocellulose lifts are done. Poly A^+ mRNA is isolated from each cell population, and ^{32}P-labeled single stranded cDNA is synthesized. The duplicate filters are then hybridized with one of the two pools of ^{32}P-cDNA. Differences in hybridization intensity of a particular cDNA reflect differences in the abundance of the corresponding mRNA between the two populations of cells.

Subtractive hybridization has also been used alone or in combination with differential screening to identify growth factor-regulated genes. Single-stranded cDNA is synthesized from one of the two cell populations, and is hybridized to the mRNA of the second population. This mixture is then passed over a column of hydroxyapatite, which preferentially binds double stranded nucleic acids. The flow-through is thus enriched for sequences present in greater abundance in one cell population.

Cochran et al (1983) as well as Nathans and coworkers (Lau and Nathans, 1985; Linzer and Nathans, 1983) used differential screening to identify specific, novel mammalian genes that were induced by growth factors. Like the proto-oncogenes, the PDGF

induced transcriptional activation of these genes was not dependent on new protein synthesis. Treatment of the cells with cycloheximide in the presence of PDGF or serum led to the superinduction of their mRNA levels, due to both prolonged transcription and increased stabilization of the mRNAs. These genes were thus referred to as "immediate early", by analogy to the immediate early genes of viruses whose activation upon entry into cells does not require new protein synthesis. A functional analogy exists as well, since these immediate early genes determine subsequent waves of gene expression and thus cellular function.

Many immediate early genes have been identified by using differential screening and subtractive hybridization, or by analyzing the expression pattern of previously identified genes (Herschman, 1991). These genes represent several categories of proteins. Several families of transcription factors have been identified. These include the protooncogenes c-*fos* (and its related genes fosB, fra-1 and fra-2), c-*myc*, c-*rel* , c-*jun* and *jun*B. Serum response factor, srf, which functions in the transcriptional activation of the immediate-early genes, is also induced by mitogenic stimulation. *Nur* 77/NGF1B is a member of the steroid receptor family. Two zinc finger proteins, *egr*-1/*krox*24/NGF1-A/*Zif*-268/TIS-8 and krox-20/egr-2, were isolated by several groups. A putative transcriptional inhibitor c25, and an uncharacterized family of transcription factors TIS 11, TIS 11b, TIS 11d have also been identified. Immediate-early genes have also been found to encode the structural proteins β-actin, fibronectin, the fibronectin receptor, an actin associated protein p27, tropomyosin (Ryseck et. al, 1989) and thrombospondin (Janat and

Liau, 1992), the cytokines *JE* and *KC* (Cochran et al 1983), the clotting initiator tissue factor, a ras-related gene *rho*B, the serine/threonine protein kinases sgk (Webster et al, 1993) and Snk (Simmons et al, 1992), a glucose transporter, and prostaglandin G/H synthase/cyclooxygenase (Fletcher et.al.,1992).

Following the activation of the immediate early genes and preceding the onset of DNA synthesis, a set of "delayed early response genes" is expressed. The induction of these genes is blocked by cycloheximide, indicating that synthesis of the immediate early transcription factors is required. Using a combination of subtractive hybridization and differential screening, Lanahan et al (1992) performed the most complete analysis of the delayed early response of BALB/C 3T3 fibroblasts to serum stimulation. Among the clones related to known cDNA sequences were genes that functioned as both permissive to proliferation and DNA synthesis, such as adenine phosphoribosyltransferase, as well as those whose functions were regulatory, such as cyclin CYL1. These genes were activated in a variety of cell types following mitogenic stimulation, including T-cells treated with IL-2 and in rat liver cells following partial hepatectomy. In contrast to the immediate early genes, these delayed early genes were not activated in hippocampal cells by depolarization or in PC12 cells during NGF-induced differentiation. Feng and Liau (1993) identified a novel delayed early response gene, PS4, using differential screening of serum stimulated rabbit vascular smooth muscle. PS4 expression peaked 4 hrs after serum stimulation of quiescent VSMC, and was found at much higher levels in fetal than in adult tissues.

The activity of growth inhibitors is also critical to the control of cell proliferation. The transition from G0 to G1 requires the repression of growth inhibitors. Schneider et al (1988) have identified a set of genes whose expression is growth arrest-specific (*gas* genes), and is rapidly decreased following mitogenic stimulation. Overexpression of *gas*1 blocks cell cycle progression (Del Sal et al, 1992), demonstrating the functional importance of repressing *gas*1 during the exit from G0. Growth inhibitory molecules are also induced during the primary response to mitogens. These include a protein tyrosine phoshatase (Charles et al, 1993; Noguchi et al, 1993), interferons, and (2'5')-oligoadenylate synthetase (Zullo et.al., 1985). These factors may modulate the activities of the growth promoters to maintain controlled cell growth.

Analysis of the immediate early gene response in T-cells, liver cells, and fibroblasts reveals that approximately one third of these genes are restricted in their expression (Zipfel et al, 1989; Mohn et al, 1991). At the time of initiation of this project, the study of growth factor regulated genes in smooth muscle had been limited to genes identified in other cell types (primarily 3T3 fibroblasts). To identify additional genes either positively or negatively regulated during the G0-G1 transition of VSMC, differential screening was done. A novel immediate-early gene, SM-20, was identified. Unlike other immediate-early genes examined in VSMC, SM-20 was not expressed in fibroblasts. This thesis details the characterization of SM-20.

Chapter 2: Materials and Methods

Cell Culture

VSMC were isolated from the thoracic aortas of 200-300g adult male Sprague-Dawley rats by enzymatic dissociation. VSMC were serially passaged in Dulbecco's modified Eagle's medium (DME-M) supplemented with 10% heat-inactivated calf serum (Sigma) on 100 mm dishes (Falcon). Cells in passages 11-15 were used for all experiments.

Human VSMC were isolated from normal human aortas harvested from explanted hearts at the time of cardiac transplantation, and cultured in 10% fetal calf serum. Cells were made quiescent by 48hr incubation in serum free medium (DME-M supplemented with 25μg/ml bovine serum albumin (BSA), 40μg/ml ascorbic acid, 5μg/ml transferrin, and 5.75μg/ml insulin).

Mouse BALB/3T3 clone A31 fibroblasts were obtained from American *Type Culture* Collection (ATCC) and grown in 10% calf serum/DME-M.

C2C12 myoblasts were obtained from Dr. Stave Kohtz (Mount Sinai Medical Center, New York) and were cultured in 15% fetal calf serum/DME-M. To perform immunocytochemical analysis, 2.5 X 10^4 cells were plated into each well of a 12-well culture dish. To induce differentiation, the cells were washed twice with PBS, and incubated in 3% horse serum for 4 days.

Total RNA from PC12 cells was generously provided by Dr. Steven Salton (Mount Sinai Medical Center, New York).

Rat 6 fibroblasts were obtained from Dr. Robert Krauss (Mount Sinai Medical Center, New York), and were maintained in DME-M, containing 1.2g sodium bicarbonate per liter, supplemented with 10% heat inactivated calf serum.

Synthesis of a rat VSMC cDNA library

mRNA was isolated from VSMC treated with PDGF for 3 hrs. An oligo dT-primed cDNA library was generated in λZapII by Dr. Claire-Lise Rosenfield according to the manufacture's protocol (Stratagene).

Differential screening

The cDNA library was plated by adding 500 plaque forming units (pfu's) to a 200 µl suspension of E. coli strain XL1-Blue (Stratagene), $O.D._{600}$=1.0. After a 15 min incubation at 37°C, 4 ml of molten top-agarose were added, and the mixture was poured onto a 100 mm plate. Duplicate nitrocellulose lifts were done. Alkaline denaturation of the DNA was achieved by placing the filters plaque-side up onto Whatman paper soaked in 0.5M NaOH, 1.5M NaCl for 5 min., followed by neutralization by 2 10-min incubations in 0.5M Tris, pH 8.0, 1.5MNaCl. The filters were air dried and then baked at 80°C for 1 hr. The filters were wetted with 2XSSC (1X= 0.55mM sodium citrate, 0.15M sodium chloride) and prehybridized overnight at 42°C in 5XSSC, 0.025 M sodium phosphate, pH6.5, 0.1%SDS, 250 µg/ml calf thymus DNA, 5XDenhardt's solution, 50% formamide and 8 ng/ml oligo (dT). ^{32}P-labeled single stranded cDNA probe was

synthesized by oligo dT-primed reverse transcription of mRNA iolated from quiescent and 3 hr PDGF-treated VSMC. Filters were hybridized overnight with 10^6 cpm/ml of cDNA probe at 42°C in 5XSSC, 0.05 M sodium phosphate, pH 6.5, 0.1%SDS, 100 µg/ml calf thymus DNA, 1 X Denhardt's solution, 50% formamide and 5 X 10^6 cpm of ^{32}P-labelled first strand cDNA per filter. Final washes were done using 2 X SSC, 0.1% SDS at 65°C. The time of exposure to autoradiographic film was adjusted such that the intensity of the majority of plaques was within the linear range of the film. Clones displaying >5 fold difference in signal intensity between the quiescent and PDGF-stimulated ^{32}P-labeled cDNA were collected from the plates by boring out the piece of agar with a pipette. To purify the differentially hybridizing clones to homogeneity, two additional rounds of screening were done. Plasmids consisting of pBluescript SK+ containing the cDNA insert were rescued from the phage clones according to the manufacture's protocol (Stratagene).

RNA isolation

A) Cultured Cells

Cells grown on 100 mm dishes were washed twice with 6 ml of PBS, and then lysed in 4 ml/plate of 4M guanidine, 0.5% N-laurel sarcosine, 0.025 M sodium citrate, pH 7.0. Chromosomal DNA was sheared by repeated passage of the lysate through a 21 gauge needle. 8 ml of this lysate were then layered onto 3.5 ml of 5.7M CsCl, 0.1 M Ethylenediaminetetraacetic acid (EDTA) and centrifuged at 10^5g for 16 hrs. The supernatant was removed by vacuum

suction, and the RNA pellet was resuspended in 400 µl of diethyl pyrocarbonate (DEPC)-treated H_2O. The RNA was precipitated by the addition of 0.25M sodium acetate, pH 5.2, and 1ml of ethanol, and a 1 hr incubation at -20°C. After centrifugation at 4°C for 15 min at 10,000 g, the supernatant was removed, and the pellet was washed with 80% ethanol to remove contaminating salts. The pellet was then resuspended by an overnight incubation in 250 µl of H_2O at 4°C. The concentration of RNA was determined by the optical density at 260nm. The pellet was reprecipitated, and then resuspended in H_2O at a concentration of 2µg/ml.

B) Rat tissues

Adult male Sprague-Dawley rats were sacrificed by lethal injection of ketamine. Organs were removed, rinsed in PBS, frozen in liquid nitrogen in polypropylene tubes, and stored at -80°C. The samples were chopped into small pieces, placed into 4M guanidine, 0.7% β-mercaptoethanol, 0.025 M sodium citrate, pH7.0, and then homogenized. N-laurel sarcosine was added to a final concentration of 0.5%. To remove residual pieces of tissue, the suspension was centrifuged at 2,000 rpm for 30 min. The aqueous phase of the supernatant was then treated identically to the guanidinium lysate from cultured cells.

Northern Blot analysis

10μg of total RNA was mixed with 3 vol. of loading buffer (final concentrations 48% formamide, 6% formaldehyde, 15 mM MOPS, 0.75mM EDTA, 4% glycerol, 0.075% bromphenol blue, 0.075% xylene cyanol, 0.075% sodium dodecyl sulfate (SDS)), heated to 65°C for 2 min, and loaded onto an agarose gel (0.9 % agarose, 10mM 4-morpholinepropanesulfonic acid (MOPS), 0.5mM EDTA, 3% formaldehyde, 100 ng/ml ethidium bromide). Following electrophoresis, formaldehyde was removed from the gel by two 30-min. washes in 10 X SSC. Overnight transfer of the RNA to nitrocellulose (BA-S NC; Schleicher and Schuell) was done using 10 X SSC, and the filter was then baked in a vacuum oven at 80°C for 1 hr. A polaroid picture of the U.V.-illuminated nitrocellulose filter was taken to verify equal amounts of ribosomal RNA loaded per lane. The filter was prewetted in 2 X SSC, and then prehybridized overnight at 42°C in 25 ml prehybridization buffer (5XSSC, 0.025 M sodium phosphate, pH6.5, 0.1%SDS, 250 μg/ml calf thymus DNA, 5XDenhardt's solution, 50% formamide), followed by overnight hybridization with 1.5 X 10^6 cpm/ml at 42°C in 20 ml of hybridization buffer (5XSSC, 0.05 M sodium phosphate, pH6.5, 0.1%SDS, 100 μg/ml calf thymus DNA, 1 X Denhardt's solution, 50% formamide) containing 30 X 10^6 cpm of ^{32}P-labeled cDNA probe. Final washes were performed using 0.5 X SSC/ 0.1% SDS. Equal loading of RNA was verified by inspection of the 28S and 18S rRNA, and by additional hybridization of the filters with a cDNA probe derived from a 3kb cDNA sequence of rat fibronectin, which is constitutively expressed and not apparently regulated in rat VSMC

culture. Autoradiograms were scanned using a Silverscanner (Lacie, Ltd., Beaverton, OR) and Adobe Photoshop 2.5 software (Adobe Systems, Inc., Mountain View, CA). Two dimensional densitometry, using 256 grayscales, was done using Image 1.43 software (Wayne Rasband, National Institutes of Health, Bethesda, MD.). Samples were normalized to the 28S rRNA or fibronectin hybridization intensity.

Generation of ^{32}P-labeled cDNA probe

The cDNA insert of pSM-20/2 was isolated by digestion with EcoR1 followed by electrophoresis on a 0.7% low-melt agarose gel. The band corresponding to the 2.5 kb insert was excised and diluted with 3 vol of TE, pH 7.5, to attain a final concentration of 1 ng/µl. To radioactively label this fragment, 25 µl was removed, boiled for 5 min to denature the DNA, and then quick-cooled on ice for 30 sec. A 50 µl solution containing 40 µM each of dGTP, dTTP, and dATP, 2 µM ^{32}P-dCTP, 3.75 µg random hexamers, 67mM KPO_4 (ph 7.5), 6.6 mM $MgCl_2$, 1mM DTT, 10 units Klenow DNA polymerase, and 25 ng of DNA template was prepared. After a 1 hr incubation, unincorporated nucleotides were removed using a G-50 sephadex spin column.

Genomic Southern Blot Analysis

Rat VSMC were grown to post-confluence, washed with PBS, lysed in 2ml/100mm plate of buffer (1mM EDTA, 1%SDS, and 10mM Tris, pH 8.0), and scraped into polypropylene tubes. Proteinase K was added to a final concentration of 100µg/ml and the mixture was

incubated overnight at 37^{0}C. Phenol extraction was done twice, followed by dialysis against TE (10mM Tris, pH 8.0, 1mM EDTA) overnight. Genomic DNA was ethanol precipitated, and resuspended by incubation in TE for 24hrs at 4°C. 400µl overnight digests containing 100 units of restriction enzyme and 10µg of RNAse A were performed. The resulting products were size fractionated by electrophoresis through a 0.6% agarose gel. Denaturation of the DNA by 20 min incubation of the gel in 0.5N NaOH/ 1.5M NaCl was followed by neutralization in 0.5M Tris, pH 8.0/ 1.5M NaCl. The DNA was transferred to nitrocellulose with 10 X SSC, and hybridized to a ^{32}P-labeled probe generated by random priming of the 2.5 kb insert of SM-20/2 (see figure 2A).

Sample Preparation for *in vivo* analysis of SM-20

A) Human coronary arteries

Samples of human coronary arteries were obtained from autopsy specimens provided by Dr. John Fallon.

B) Rat aorta

400g adult male Sprague-Dawley rats were anesthetized with ketamine, and perfused via the descending aorta with 100 ml of PBS, followed by 100 ml of 4% paraformaldehyde/ PBS. The aortas were removed and placed in 4% parafomaldehyde/PBS at 4°C for 2 hrs, folllowed by 15% sucrose/PBS for 2 hrs, and then 30% sucrose/PBS for 2hrs. The aortas were embedded in O.C.T. (10.24 % polyvinyl alcohol, 4.26% polyethylene glycol; Tissue-Tek) and quick-frozen in a dry ice ethanol bath before being stored at -80°C. Cryostat sections

were placed onto Superfrost plus slides (Fisher) and allowed to air dry and used for *in situ* hybridization or immunohistochemistry.

In situ hybridization

Production of the riboprobe: A 492 bp fragment extending from the Pst1 site at base 2348 to the EcoR1 site of clone SM-20/2 was subcloned into pBluescript KS+ cut with Pst 1/ EcoR1. The resulting plasmid was then cut with EcoR1 or Pst1 in order to generate sense and antisense riboprobes using the T7 and Sp6 RNA polymerases, respectively. After 45 minutes of this polymerase reaction, the probe was gel purified. 1.5 volumes of a formamide loading buffer (80% formamide, 0.1% xylene cyanol and bromphenol blue, 5mM EDTA) was added to the solution which was then heated to 85°C for 5 min and quick-cooled on ice before loading onto a 4% polyacrylamide gel. After electrophoresis for 1.5 hr, the bands containing the full-length probes were excised. The probes were then eluted for 4 hrs at 37°C into 1%SDS/2M ammonium acetate/1mM EDTA and then ethanol precipitated. The probe was resuspended at a concentration of 1.5 X 10^5 cpm/µl in hybridization mix (20% deionized formamide, 10% dextran, 10mM sodium phosphate, pH 8.0, 0.5M NaCl, 8mM Tris, pH 8.0, 2 mM EDTA, 0.8 X Denhardt's solution, 0.4 mg/ml yeast tRNA, 80 µg/ml poly A, 50 mM DTT).

Hybridization: The sections were rehydrated in PBS and then treated with proteinase K (3 µg/ml in 50mM Tris, pH 7.5, 5 mM EDTA) at 37°C for 10 min. A wash was done with PBS containing

glycine (3 mg/ml), followed by three washes with PBS. Acetylation of positively charged residues to reduce nonspecific binding of the riboprobe was done by a 5 minute incubation in 0.1 M triethylamine (TEA), followed by the addition of acetic anhydride to a final concentration of 0.25% for 10 min. The slides were rinsed twice for 5 min in 2X SSC and then dehydrated by sequential 5 min ethanol rinses of 30%, 60%, 80%, 95%, and 100 %, followed by air drying. 20µl of hybridization mix was then added to each section and incubated at 45°C for 2 hr for the prehybridization. 2 µl of the probe (300,000 cpm) was then added to each section, and hybridized overnight at 45°C in a moist chamber. Two 15-min washes of 2 X SSC, 50% formamide, 0.1% β-mercaptoethanol were done at 50°C. The slides were then treated for 30 min with 20 µg/ml RNAse A in 0.5 NaCl, 10 mM Tris, pH 8 for 30 min at 37°C. Two 15-min washes were again done in 2 X SSC, 50% formamide, 0.1% β-mercaptoethanol at 50°C. Two final 30 min washes were done 0.1 X SSC, 1% β-mercaptoethanol at 50°C. Dehydration of the sections was done by 2 min incubations in 0.6 M NaCl in 30% ethanol, 0.6 M NaCl in 60% ethanol, then 80%, 95%, and finally 100% ethanol followed by air drying. The slides were dipped into autoradiographic emulsion (KODAK) prewarmed to 45°C and then air dried overnight. Exposure was done for 4 wks in a light-tight slide box containing dessicant at 4°C. The slides were then incubated in developer solution (KODAK) for 2.5 min at 16°C, rinsed for 30 sec in tap water, dipped into fixer (KODAK) for 3 min., and rinsed in tap water for 10 min. Sections were counterstained with hematoxylin, rinsed in water, dipped into 0.1% ammonium hydroxide for 10 seconds, and then rewashed in

water. Dehydration was done by 2 min incubations in 30%, 60%, 80%, 95%, and 100% ethanol, followed by 50% ethanol/50% xylene, and finally 100% xylene. Mounting media and coverslips were added, and pictures were taken in dark field and bright field.

Immunohistochemistry

Immunostaining was perfomed using the Zymed Histostain SP kit according to the manufacturer's protocol. Sections were hydrated with PBS and endogenous peroxidase activity was blocked by a 45 second treatment with a periodic acid solution (peroxo-block, Zymed). Blocking of non-specific protein binding sites was done using 10% goat serum for 30 min. The primary antibody was diluted 25 fold in this blocking solution, and incubated overnight at 4°C. The sections were washed in PBS three times before incubation with a biotinylated secondary antibody (goat anti-rabbit) for 25 minutes. PBS washes were followed by incubation with avidin-conjugated horseradish peroxidase for 25 minutes. Following three final PBS washes, a substrate-chromogen mixture consisting of amino ethyl carbonate (AEC) or diaminobenzidine (DAB), 0.06% hydrogen peroxide, and Tris buffer was added for 10 minutes. The slides were washed with tap water and counterstained with hematoxylin.

Production of SM-20 immunogen

cDNA clone SM-20/2 was digested with Xho II, and the resulting fragment extending from base 568 to 1321 was ligated into the pet-3b (Novagen) bacterial expression vector and sequenced. This new vector, pETSM-20, encoded a fusion protein consisting of the first 11 amino acids of the phage T7 gene *10* capsid protein, an arginine residue, and then residues 126 to 355 of the SM-20 putative peptide sequence. Expression of the mRNA transcript for this peptide is under the control of the phage T7 RNA polymerase promoter. This vector was transformed into the E. coli strain BL21 which contains the T7 polymerase gene under the control of the lac Z promoter. A single bacterial colony from this transformation was used to inoculate 200 ml of LB broth. The culture was grown to log phase, and production of the T7 polymerase was induced by the addition of 0.5 mM Isopropylthiogalactoside (IPTG). After 2 hrs 1.5 ml was removed and used for mini prep analysis to ensure that the majority of the culture retained the intact plasmid. The rest of the bacteria was collected by centrifugation at 3,000 rpm for 10 min.

A bacterial extract enriched for the SM-20 peptide was generated by passing the pellet through a series of resuspension and re-pelletting steps using solutions with increasing stringency for solubilization of proteins. The bacterial pellet was first resuspended in 8 ml of TEN (50 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl) containing 10 µg of lysozyme. After 30 min of gentle shaking at room temperature, the suspension was centrifuged at 4,000 rpm for 10 min. The supernatent was saved and the pellet was resuspended in 8 ml of TEN containing 0.1% deoxycholic acid, 10 mM $MgCl_2$, and

10 µg/ml DNAse 1. After centrifugation the pellet was resuspended in 0.5% triton/ TEN by sonication, and then in 0.5% Triton/PBS by sonication. The final insoluble pellet was solubilized in SDS buffer (2%SDS, 100mM Tris pH 6.8, 100mM DTT, 10% glycerol) at 95°C for 10 min.

To determine which of the fraction(s) contained the fusion protein, 30 µl of each fraction as well as the final pellet was loaded onto a 12% SDS- polyacrylamide gel. Fractions prepared from transformed culture of BL21 which had not been induced by IPTG were also run on this gel. Staining with Coomassie blue revealed that the SDS solubilized fraction of the IPTG induced culture contained an extremely abundant protein which migrated below the 31 kD marker. This indicated that the 28 kD fusion protein had been successfully overexpressed. As seen in figure 13, most of the SDS fraction consisted of the SM-20 fusion protein. Many of the other BL21 proteins were present in the fractions solubilized under the earlier, less stringent washes.

One hundred and twenty five micrograms of the enriched extract was loaded onto each of two 12% preparative SDS-PAGE (the top of the stacking gel was a single flat surface with no indentations for individual lanes). After separation of the proteins was complete, a strip from the side of each gel was removed and acid stained with Coomassie Blue to reveal the position of the expressed protein. This portion of each gel was then excised and combined into a single syringe with an equal volume of an aluminum-based adjuvant (Imject Alum, Pierce). A suspension of protein/acrylamide in the adjuvant was created by repeated passage through an 18 gauge

needle. This mixture was then injected subcutaneously into 4 sites on each of two 3 kg white New Zealand rabbits. After 14 days, 20 ml of blood was drawn from each rabbit and allowed to clot. The serum was then collected and affinity purified. Injections were repeated every six weeks.

Affinity purification of SM-20 antisera

In order to purify antibodies that were directed specifically against the putative SM-20 peptide sequence, two affinity columns were generated. 1gram of CNBR-activated sepharose (Pharmacia) was resuspended and swollen in 1mM HCl for 15 min., and then rinsed 3 times in 1mM HCl. The beads were then equilibrated in coupling buffer (0.1M $NaHCO_3$, pH 8.3; 0.5M NaCl) for 2 hrs. 10mg of either the SM-20 immunogen or a protein extract prepared from BL21 transformed with pET3b vector was then added to the beads and incubated for 2 hrs. Excess ligand was removed by washing in coupling buffer. Remaining active sites were saturated with 0.2M glycine/ coupling buffer. The beads were washed with 3 cycles of alternating pH. Each cycle consisted of a wash with acetate buffer (0.1M acetate, pH 4.0, 0.5M NaCl), followed by Tris buffer (0.1M Tris, pH 8.0, 0.5M NaCl). The columns were equilibrated with 100 ml PBS. Total serum was diluted with 3 volumes of PBS. The diluted serum was first passed through the BL21 column three times to remove anti-E. coli antibodies. The flow-through was collected and added to the SM-20 column. SM-20 antibodies were eluted using 0.1M glycine pH 3.0, and the solution was neutralized with 0.5M sodium

phosphate, ph 7.7. This solution was then concentrated to 10% of the original volume of the serum (approximately 0.9ml) by vacuum filtration. BSA was added to a final concentration of 3% to stabilize the antibodies, and the solution was dialyzed against 300 ml of PBS overnight. The antisera was then aliquoted and stored at -80°C. This procedure was performed on each of the bleeds, as well as on a sample of the pre-immune sera.

Titration of the antisera

Enzyme-linked immunosorbant assays (ELISA) were performed to titre the affinity of the purified antisera for the SM-20 sequence. 100 ng of the SM-20 immunogen was adsorbed onto PVC microtitre wells by adding 50 µl of a 2mg/ml.PBS solution to each well. After a 2 hr incubation at room temperature in a humid chamber, unbound protein was removed by 2 washes with PBS. Remaining protein-binding sites were saturated with 3% BSA /PBS. The wells were washed twice with PBS. Two-fold serial dilutions of the affinity purified antibody were made in 3%BSA/ PBS and ranged from 1:2 to 1:1000 fold dilutions. Unbound antibody was removed by four washes of PBS. A goat anti-rabbit IgG-alkaline phosphatase conjugate (Boehringer Manheim) was diluted 1:7500 in 3%BSA/ PBS and added to the wells. After a two hr incubation at rooom temperature in a humid chamber, the unbound antibody was removed by four washes with PBS. Detection of bound antibody was performed by adding a substrate solution consisting of 0.33 mg 4-Nitro blue tetrazolium chloride (NBT), 0.17mg 5-Bromo-4-chloro-3-

indoyl-phosphate (BCIP) per ml of alkline phosphatase buffer (100mM NaCl, 5mM $MgCl_2$, 100mM diethanolamine) (Boehringer Manheim). Reactions were allowed to proceed for approximately 2 hrs and then observed for the intensity of the color product.

Immunocytochemistry

VSMC were grown on 12 well dishes to 80% confluence and then made quiescent by a 48 hr incubation in 0.3% calf serum. Cells were washed three times in PBS and then fixed in 4% paraformaldehyde/PBS for 10 min. Following washes in PBS, the cells were permeabilized with 0.2% triton/PBS for 2 min and then washed with PBS. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide for 10 min. After PBS washes, nonspecific protein binding sites were blocked with 10% goat serum for 1 hr. The affinity purified antisera was then diluted 100 fold in 10% goat serum, and 250 µl was placed into each well and allowed to incubate overnight. Detection using AEC as the substrate was done as described under "Immunohistochemistry".

Western Blot analysis

VSMC were grown to 80% confluence on 100mm dishes and then made quiescent by a 48 hr incubation in 0.3% calf serum. Extracts containing total cellular proteins were prepared as follows. 300 µl of SDS buffer (2%SDS, 100mM Tris pH 6.8, 100mM DTT, 10% glycerol) was added to each plate, the lysate was scraped into a

microcentrifuge tube, and denatured by heating at 95°C for 10 min. Quantification was done using a standard Bradford assay (Bio-Rad). 25 µg of total VSMC protein was loaded into each well of a 12% polyacrylamide gel (4% stacking gel). Electrophoresis and transfer to nitrocellulose were done according to the manufacturer's protocol (mini blot system, Bio-Rad). Following transfer, the polyacrylamide gel was stained with Coomassie Blue to visualize the remaining proteins and verify equal loading of proteins among lanes. Detection of the transferred SM-20 protein was done using an enhanced chemiluminescent system (Amersham) according to the manufacturer's protocol. The nitrocellulose sheets were blocked with 5%BSA/TBST (20mM Tris, pH 7.6, 137mM NaCl, 0.1% Tween 20) and incubated for 1 hr. with the primary antibody (affinity purified immune or preimmune) diluted 1/200 in the blocking solution. The filters were washed in TBST before addition of horseradish peroxidase conjugated donkey anti-rabbit antibody (Amersham) diluted 1/3 X 10^4 in the blocking solution. After washing in PBS, the chemiluminescent solutions were applied to the filters, which were exposed to autoradiography film (Dupont).

TCA precipitation of VSMC conditioned medium

VSMC were grown to 80% confluence in 10% calf serum, washed with PBS, and then placed in serum free medium (DME-M supplemented with 25µg/ml bovine serum albumin (BSA), 40µg/ml ascorbic acid, 5µg/ml transferrin, and 5.75µg/ml insulin) for 48hrs. The cells were washed with PBS and incubated in 6 ml of DMEM

containing 1μM isoproterenol for 6 hrs (isoproterenol raised the SM-20 mRNA to the highest levels). One ml of the conditioned medium was placed into each of six 1.5 ml eppendorf tubes. 160μl of 100% TCA was added (final concentration of 14%) to each tube, and incubated on ice for 30 min. The TCA precipitable polypeptides were pelleted by centrifugation at 10,000g for 25 min. The pellets were washed with PBS and resuspended in a total of 60 μl of Laemmli buffer. 30μl of this was loaded per lane of a SDS-PAGE for Western blot analysis.

Competitive inhibition of antisera binding

An SM-20-enriched bacterial extract (see "Production of SM-20 antisera") and a BL21 (transformed with pET 3b vector alone) total protein extract were each diluted into 100ml of PBS at a concentration of 50 μg/ml. Nitrocellulose (2 cm X 3 cm) was added to each of these solutions and incubated at room temperature for 1 hr. The nitrocellulose was removed, washed 3 times with PBS, and then the remaining protein binding sites were saturated by incubation in 3% BSA/PBS for 1 hr. 500 μl aliquots of a 1:100 dilution of the primary antibody were placed on parafilm in moist chambers. The nitrocellulose sheets adsorbed to either the BL21 or the SM-20 enriched extract were added to these aliquots. After 1 hr the antisera were collected and used as the primary antibody solution in the same protocol outlined under "Immunocytochemistry".

Chapter 3: Cloning of a Novel Mitogen-responsive Gene in VSMC

The initial aim of this project was to identify a novel gene whose mRNA levels were regulated following mitogenic stimulation of VSMC. PDGF was chosen as the mitogen for the differential screening assay for several reasons. For cultured VSMC, PDGF functions as both a competence and progression factor, thus eliciting a complete mitogenic response (Clemmons, 1984). PDGF is also a potent chemoattractant for VSMC (Ross, 1993). Therefore, genes induced during one or both of these processes, which are critical to atherogenesis, could be identified. In addition, gene regulation by PDGF *in vitro* has been found to correlate with gene activation in the vessel wall following arterial injury (Taubman, 1993a; Miano et al, 1993a and b; Marmur et al, 1993).

PDGF is a dimer composed of two subunits, A and B, which can either homo- or heterodimerize (Ross et al, 1986). Two distinct PDGF receptors have been identified, referred to as α and β. The A and B subunits bind to these receptors with different specificity. B binds the β isoform with high affinity, whereas the A subunit only binds at high concentrations. The α receptor isoform specifically binds the A subunit. Cultured VSMC express low levels of the α receptor, and respond weakly to treatment with AA homodimer. The β isoform of the PDGF receptor has been found to mediate the majority of the mitogenic response in rat arterial smooth muscle (Williams, 1989); therefore recombinant BB homodimer (c-sis) was used.

In order to identify immediate early as well as delayed early response genes, we based our differential screening on 3 hrs of PDGF stimulation. Poly A+ mRNA was isolated from quiescent and PDGF-treated VSMC. An oligo dT-primed cDNA library was synthesized from a portion of the PDGF-treated mRNA. The quiescent and PDGF-treated mRNA were then each used as templates for an oligo dT-primed ^{32}P-labeled single stranded cDNA synthesis reaction. The library was plated at low density (approximately 500 plaques per 100mm plate), and duplicate nitrocellulose filter lifts were made. Each filter was then hybridized with one of the two pools of ^{32}P-cDNA. Approximately 4 X 10^4 plaques were screened, and 50 clones demonstrating differential hybridization were purified to homogeneity. The cDNA inserts were rescued into the pBluescript SK+ vector and were ^{32}P-labeled by random primed cDNA synthesis. Northern blot analysis using these cDNA probes demonstrated that 5 of the clones corresponded to growth factor-regulated mRNA species. Four of the clones, which represented mRNA species increased 3 hrs after mitogenic stimulation, were identified as sequences previously reported to the GenBank database. These included osteopontin, thrombospondin, lactate dehydrogenase, and lysyl oxidase. The fifth clone, SM-20/1, contained a 1.8 kb insert which displayed decreased hybridization to the PDGF-stimulated ^{32}P-cDNA. Using Northern blot analysis (figure 1), the cDNA probe detected a single mRNA species in quiescent VSMC (labeled "0") whose abundance was decreased in VSMC treated with 10% calf serum for 4 hrs. SM-20/1 therefore corresponded to a differentially regulated gene expressed in VSMC. The partial sequence of this clone revealed only low (<50%) homology

to sequences in the Genbank, and thus the complete sequence of the insert was determined. Low homology was again found, suggesting that the SM-20/1 sequence was novel.

The mRNA species detected by the SM-20/1 probe migrated on Northern Blot analysis (figure 1) to a position approximately equidistant between the 18S and 28S rRNA sequences. The complete mRNA length was therefore approximately 2.5-3kb in length, indicating that SM-20/1 represented a partial cDNA. The insert contained at its terminus a polyadenylation sequence, AATAAA, and a poly (A) tail (figure 2A) separated by 12 bp, suggesting that SM-20/1 contained the 3' terminus of the mRNA. Accordingly both oligo dT-primed and random-primed cDNA libraries were screened to isolate clones containing additional sequence. Three distinct clones were isolated whose 5' ends extended ~700 bp beyond the end of the 1.8 kb clone (figure 2A).

The sequence derived from all of the SM-20 cDNA clones revealed a single large open reading frame (ORF) which began at the 5' terminus (figure 2B). It was thus possible that the full length SM-20 mRNA, and most importantly the complete coding region, had not been isolated. Additional screening of cDNA libraries failed to identify clones which extended further 5'. Therefore, a rat genomic library was screened using the SM-20/2 insert as probe. Four genomic clones were isolated and digested with EcoR1. Three of the clones contained fragments which hybridized to two oligonucleotides derived from the 5' end of the cloned cDNA (nucleotides 390-409 and 548-567). One of these fragments, containing a 1.5 kb insert,

Figure 1

Figure 1. Northern blot analysis showing differential regulation of SM-20 in VSMC culture.

Rat aortic VSMC were grown to 75% confluence and then made quiescent by incubation in 0.3% serum for 48 hrs (time 0). Cells were then stimulated for 4 hours by the addition of calf serum (final concentration 10%). Total RNA was collected and 10 µg was loaded onto each lane. Blots were hybridized to the SM-20/1 cDNA insert. The positions of the 18S and 28S rRNA are indicated.

Figure 2. Analysis of SM-20 cDNA and genomic clones.

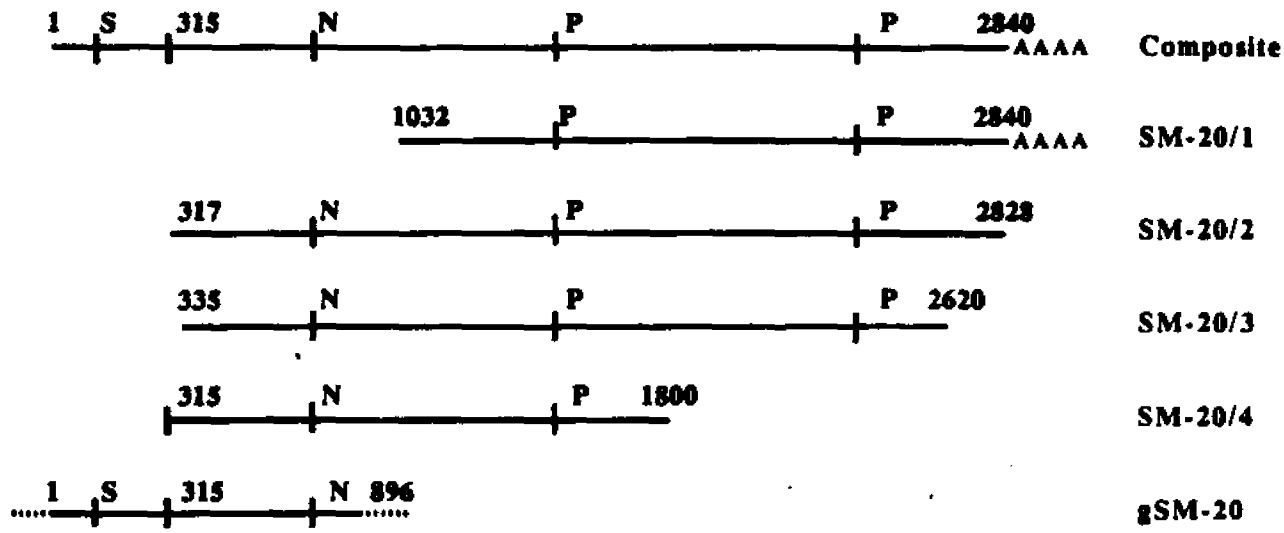
A) A map of the composite sequence derived from cDNA and genomic clones is shown on top. The initial clone identified by differential screening, SM-20/1, was used to isolate additional cDNA clones SM-20/2-4, as well as a genomic clone gSM-20. Residue 315 of the combined sequence is the most 5' nucleotide found in any of the cDNA clones. The dotted lines in gSM-20 represent additional 5' DNA not included in the combined sequence, as well as 3' DNA whose sequence diverges from the cDNA sequence and is presumably intronic.

B) Open reading frame analysis of the SM-20 sequence derived from the cDNA clones. Numbering reflects the nucleotide position in the combined sequence shown in A). Thick lines represent peptide sequences extending at least 25 residues in the three possible reading frames (the 5'-3' orientation of the SM-20 sequence was suggested by the poly A tail in clone SM-20/1. Line 1 contains the major open reading frame, which begins at the 5' end of the cDNA clones sequence and extends 939 bp.

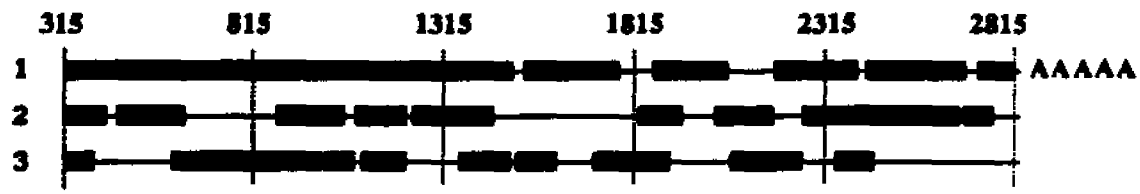
C) Open reading frame analysis of the composite sequence. An open reading frame beginning within the sequence isolated from the genomic DNA is in-frame with the major open reading frame identified in B)..

Figure 2

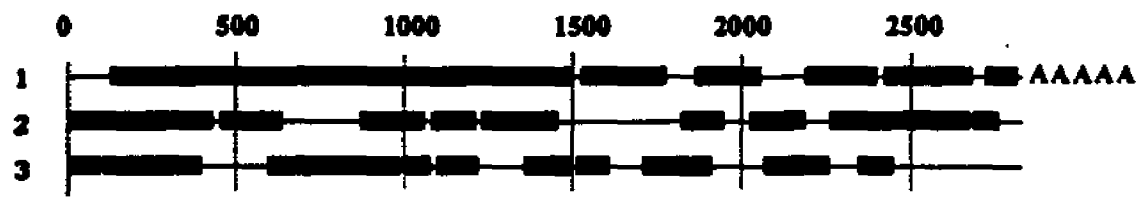
A.


1 S 315 N P P 2840 AAAA
Composite
1032 P P 2840 AAAA
SM-20/1
317 N P P 2828
SM-20/2
335 N P P 2620
SM-20/3
315 N P 1800
SM-20/4
1 S 315 N 896
gSM-20

B.


315 815 1315 1815 2315 2815
1
2
3
AAAAA

C.


0 500 1000 1500 2000 2500
1
2
3
AAAAA

was subcloned into pBluescript KS+ to generate plasmids gSM-20 (figure 2A). This insert contained 581 bp which matched the 5' terminus of the cDNA clones, and extended an additional 315 bp upstream.

Analysis of the combined sequence (figure 3) derived from the genomic and cDNA clones revealed that the open reading frame extended an additional 123 bp from the 5' end of the cloned cDNA to a methionine at base 191 (figure 2C). The nucleotide sequence from 182 to 194 was consistent with the consensus sequence for efficient translation initiation sites developed by Kozak (1989) as seen below:

```
     SM-20:  GGCGGGACCAUGA
             | ||  |||
Consensus:   GCCGCC(A/G)CCAUGG
```

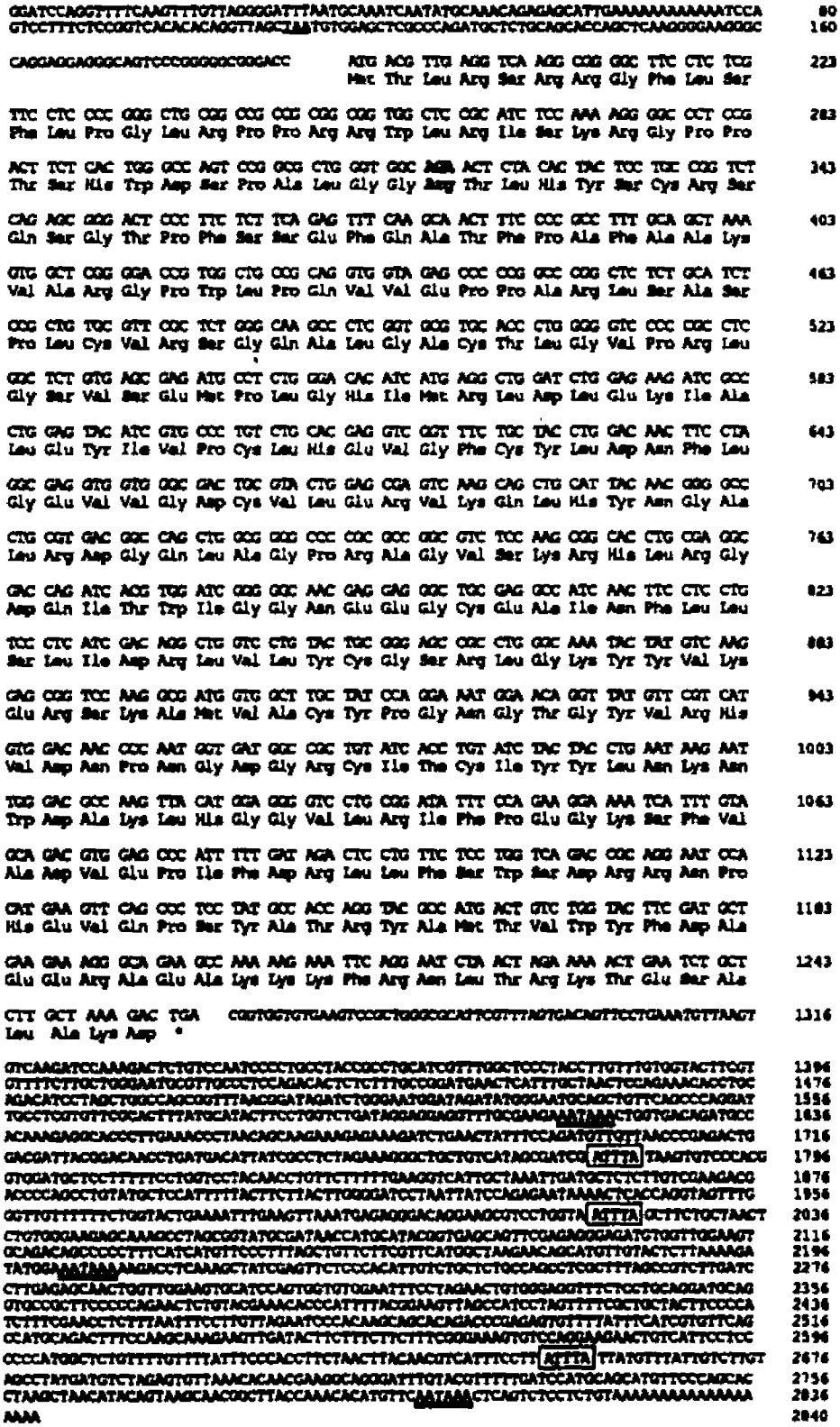
6/10 bases around the AUG were identical to the consensus, and most importantly there was a purine in position -3 (and in particular an adenine residue, which is the most commonly observed base in this position). An in-frame stop codon was located 81 bp upstream of this methionine.

To demonstrate that the sequences derived from the genomic DNA clones are present within the SM-20 mRNA, PCR analysis was performed. An oligonucleotide which corresponds to nucleotides 321-340 of the composite sequence, and is within the cloned cDNA sequence, was used to prime the reverse transcription of VSMC total RNA. The product was then used as the template for PCR using this oligonucleotide and an oligonucleotide corresponding to bases 75-94, which is upstream of the in-frame stop codon (figure 4B). Southern

Figure 3. Sequence analysis of SM-20.

The "combined" SM-20 nucleotide sequence is shown, along with the putative amino acid sequence deduced from the open reading frame (figure 2C, line 1). The codon in bold type represents the 5' end of sequence contained within the cDNA clones. A stop codon located upstream of the putative amino acid sequence is underlined. The putative 3' untranslated region contains three polyadenylation consensus sequences (double underlines) and three ATTTA elements (boxed).

Figure 3



blot analysis using an internal end-labeled oligonucleotide probe (nucleotides 189-207) detected an amplification product that migrated with a molecular weight consistent with the predicted size of 265 bp (figure 4A). This product was absent when the reverse transcriptase was omitted, indicating that it was derived from cellular RNA and not contaminating genomic DNA. A product of the same size was also generated by PCR using the cDNA library as the template. In addition, a single stranded probe corresponding to nucleotides 1-327 was hybridized to the Northern Blot shown in figure 4C. This probe detected an mRNA species with the same molecular weight and pattern of regulation as detected by the 2.5 kb cDNA clone SM-20/2. These results indicate that the SM-20 mRNA extends at least through nucleotide 75 of the composite sequence, including the in-frame stop codon. This is consistent with the Met at nucleotide 191 being the start site of translation.

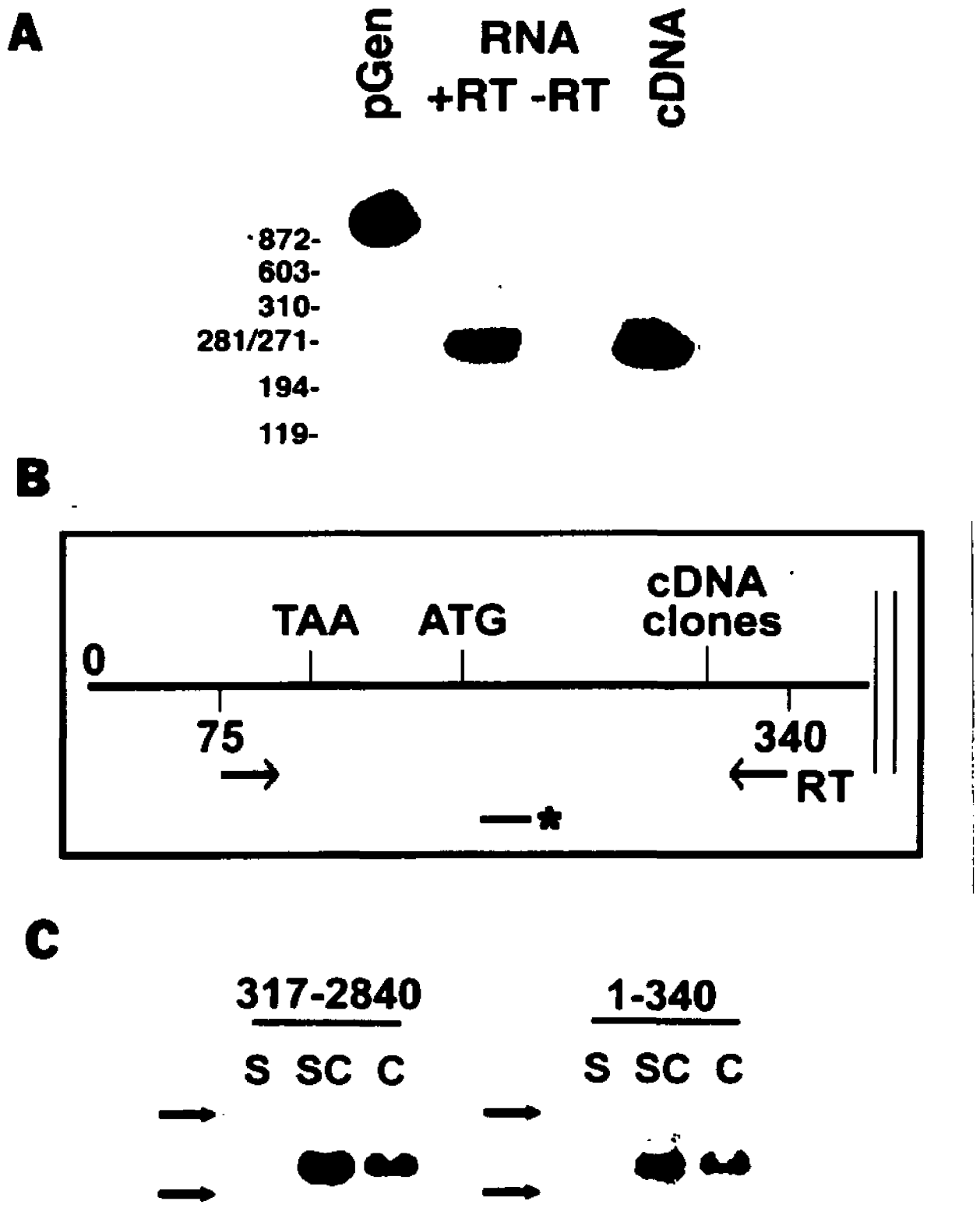
The "combined" SM-20 open reading frame extending from base 191 to 1255 encodes a 355 amino acid protein with a predicted molecular weight of 39.7 kD. This protein is predicted to be extremely basic, with a pI estimated at 9.93. Hydropathy analysis (figure 5) using the Kyte-Doolittle scale (1982) with a window size of 19 amino acids indicates a hydrophilic amino terminus of 60 amino acids, followed by a slightly hydrophobic stretch of 100 amino acids, and a predominately hydrophilic c-terminus interrupted by a 20 amino acid hydrophobic region. The absence of very hydrophobic regions suggests that the SM-20 protein does not contain a signal sequence or a transmembrane domain.

Figure 4. Verification that sequences derived from the genomic DNA cloning are within the SM-20 mRNA.

A,B) Reverse transcription-Polymerase Chain Reaction (RT-PCR) analysis: 2 μg of total VSMC RNA was added to RT reaction mixtures in which the reverse transcriptase was either present (+RT) or absent (-RT). An antisense oligonucleotide, oligo-9, corresponding to nucleotides 321-340 was then used to prime the synthesis of the first strand cDNA. 5μl of each 20μl reaction as well as $2X10^6$ pfu's of a cDNA library (cDNA) were then used as the template for PCR using oligo-9 and a sense oligonucleotide corresponding to bases 75 to 94. Amplification of the SM-20 sequence was detected by Southern blot analysis using a ^{32}P-labeled internal oligonucleotide probe corresponding to nucleotides 189-207. Plasmid gSM-20 (see figure 2A) was included in the gel as a positive control for binding of the labeled oligonucleotide.

C) Northern blot analysis: A northern blot (figure 9) used to examine the effects of cycloheximide on SM-20 mRNA regulation was first hybridized with a cDNA probe (317-2840) generated by random priming of the 2.5kb cDNA insert of clone SM-20/2. This filter was stripped and re-hybridized with a single stranded ^{32}P-labeled DNA probe corresponding to nucleotides 1-340 of the composite sequence. Shown are the lanes containing RNA samples collected after 3 hrs of treatment with serum (S), cycloheximide (C), or both (SC).

Figure 4


A
pGen
RNA
+RT -RT
cDNA
872-
603-
310-
281/271-
194-
119-
B
TAA
ATG
cDNA
clones
0
75
340
RT
C
317-2840
1-340
S SC C
S SC C

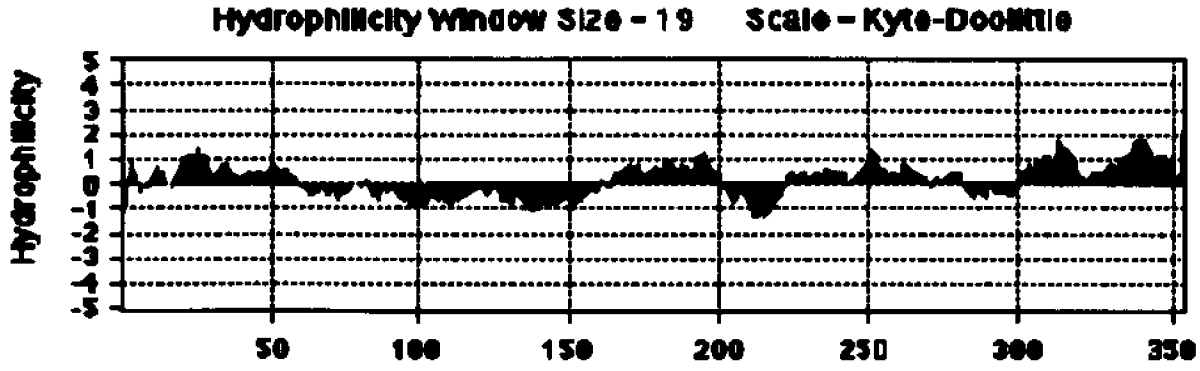


Figure 5. Hydrophilicity analysis of the putative SM-20 peptide sequence.

Numbers correspond to the amino acid position at the center of the 19 residue window.

Using MacVector software, SM-20 was analyzed for the presence of additional primary amino acid sequence motifs. The results are displayed in table 1.

Table I

SM-20 primary amino acid sequence analysis

Motifs Present (residue #)	Motifs Absent
RGD (190-192)	Basic domain-leucine zipper
N-linked glycosylation (244; 343)	Zinc finger
Acceptor sites for:	Acceptor sites for:
Protein kinase C (5)	S6 kinase
Glycogen synthase kinase (55)	cAMP-dependent kinase
Calmodulin-dependent kinase II (53; 113)	Myosin light chain kinases
	Tyrosine kinases
	Catalytic domains of:
	Serine/Threonine kinases
	Tyrosine kinases
	Serine phosphatases
	Nuclear localization signal
	Secretory signal sequence
	EF-hand*

*Determined by comparison to the consensus sequence (Moncrief et al., 1990)

The composite SM-20 nucleotide and putative peptide sequences were used to screen the GenBank and NCBI databases, respectively. Significant homology was found only to the putative amino acid sequence of an uncharacterized 389bp partial cDNA cloned from Caenorhabditis elegans (Waterston et al, 1992; Accession Number M88987). There is 70% identity and 76% similarity (including conserved substitutions) over a 50 amino acid region as indicated in figure 6.

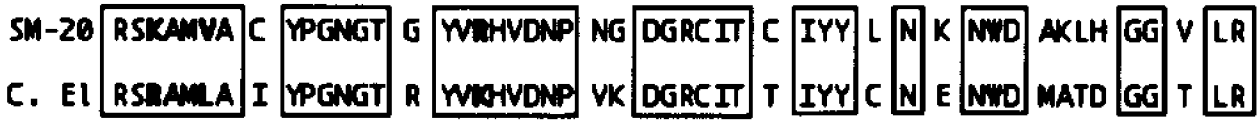


Figure 6. Comparison of the putative amino acid sequences of SM-20 and C. elegans clone M88987.

Residues 233-282 of the SM-20 putative peptide sequence are displayed along with the putative sequence encoded by M88987. Regions of identity and conserved substitutions (boldface type) are boxed. The combined SM-20 nucleotide and peptide sequences were used to screen GenBank (June, 1994).

Genomic Southern blot analysis

To determine whether there are other sequences in the rat genome related to SM-20, genomic Southern blot analysis was performed. A ^{32}P-labeled probe was generated by random priming of the SM-20/2 cDNA insert. Hybridization was done in 50% formamide/1M Na^+ and washes were done at 37°C, 0.2M Na^+ (figure 7A). Following autoradiography, the filter was then washed at the higher stringency of 65°C/0.1M Na+ (figure 7B). Both the low and high stringency conditions gave the same pattern of bands. Two or three major bands were observed from each of the digestion products. One or two faint bands were also detected. These results suggest that SM-20 is not homologous to repetitive DNA sequences.

From the above data, we can conclude that SM-20 is a novel gene that is expressed in VSMC culture. SM-20 is predicted to encode a 39.7 kD protein with little sequence homology to previously characterized proteins.

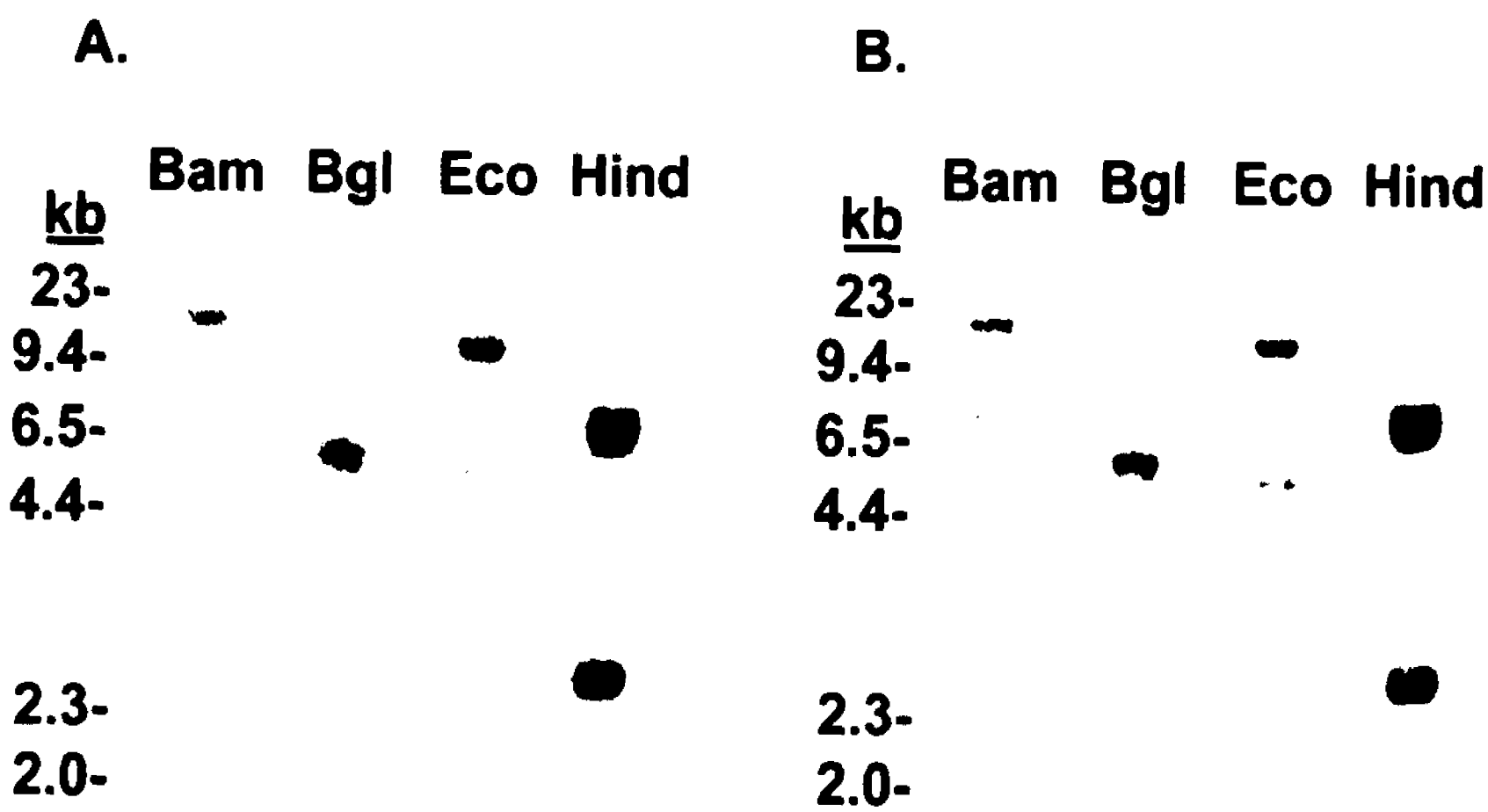


Figure 7. Genomic Southern blot analysis.

Genomic DNA was isolated from rat VSMC and digested overnight with BamH1 (*Bam*), Bgl ll (*Bgl*), EcoR1 (*Eco*), or Hind lll (*Hind*). Following transfer to nitrocellulose, the blot was hybridized (50% formamide/1M Na^+) to a ^{32}P-labeled probe generated by random priming of the 2.5kb insert of SM-20/2 (figure 2A). A) and B) represent the same blot washed at 37°C, 0.2M Na^+ (A) and then at 65°C, 0.1M Na^+ (B). There was no observable difference in the banding pattern under these conditions. The migration positions of DNA size standards are also indicated.

Chapter 4: Regulation of SM-20 mRNA

Response to Serum

SM-20 represents a novel gene expressed in cultured VSMC whose mRNA level decreased four hrs after the addition of 10% serum. To examine SM-20 regulation following serum stimulation in greater detail, Northern blot analysis was performed under a variety of conditions. As seen in figure 8, SM-20 mRNA was detected in quiescent VSMC. Following the addition of serum, SM-20 levels increased at 30 min and peaked at 1 hr (maximum fold induction=3.47 +/- 0.24, n=4). At 3 hrs, levels were at or below baseline (i.e. quiescence) where they remained for at least 16 hrs. Because the initial screening and Northern blot analysis employed 3 and 4 hr time points, respectively, the increase in SM-20 mRNA seen between 1 and 2 hrs was unexpected.

Cycloheximide Effects

The rapid induction of SM-20 mRNA levels indicated that it might be part of the primary response to growth factors. Lau and Nathans (1985) postulated that a distinguishing feature of immediate-early genes is induction by cycloheximide and superinduction by serum (or other stimuli) and cycloheximide. As seen in figure 9, Northern blot analysis demonstrated that incubation of quiescent VSMC with cycloheximide led to an increase in SM-20 mRNA at 1 hr to a level approximately equal to that following serum stimulation. Treatment of VSMC with serum and cycloheximide increased SM-20 levels to greater than that observed following

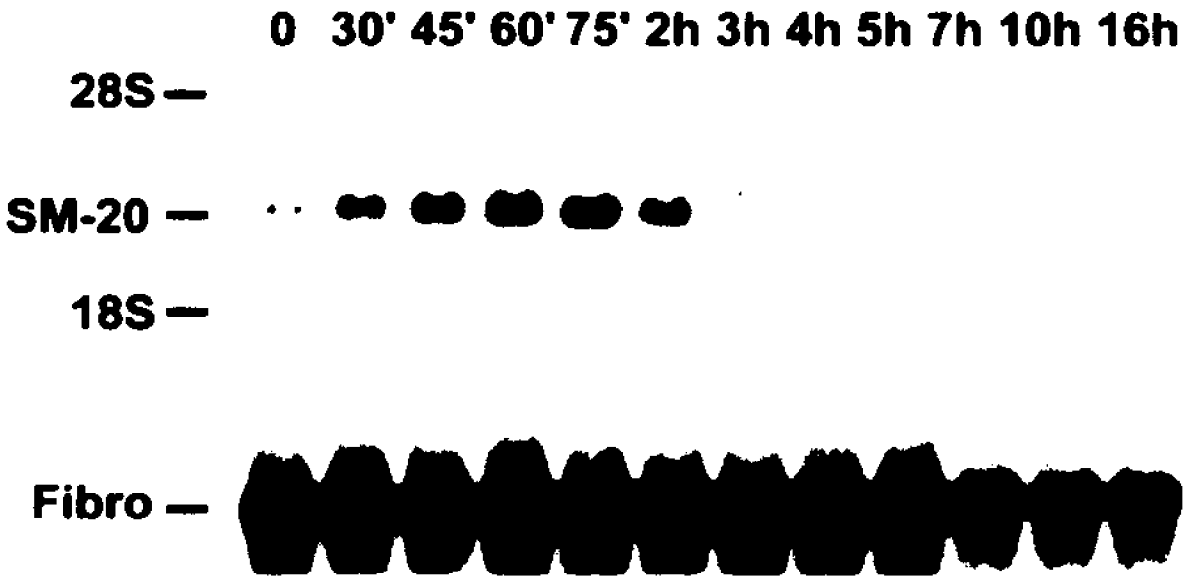


Figure 8. RNA blot analysis of SM-20 mRNA response to serum stimulation.

VSMC were incubated in 0.3% calf serum for 48 hrs to achieve quiescence (time 0). Calf serum was then added to a final concentration of 10%, and total RNA was collected at the times indicated. 10μg of each RNA sample was loaded per lane. Following electrophoresis and transfer to nitrocellulose, the filter was hybridized with a ^{32}P-labeled probe generated by random priming of the 2.5 kb insert of SM-20/2 (figure 2A). Final washes were at 65°C, 0.5 X SSC. Shown also is the hybridization signal from a rat fibronectin probe ("Fibro"), which confirmed the equal loading of RNA.

serum or cycloheximide alone. This indicates that the response of SM-20 to serum is not dependent on new protein synthesis. SM-20 is thus a member of the immediate-early gene family as defined by Lau and Nathans (1985).

Three hrs after serum stimulation, SM-20 levels returned to baseline or below. However, when cycloheximide was also present mRNA levels remained elevated even at 5 hrs (figure 9). SM-20 mRNA levels also remained elevated at 5 hrs of incubation with cycloheximide alone. This prolonged elevation of mRNA levels by cycloheximide has been consistently observed for other members of the immediate-early gene family. The difference in SM-20 mRNA levels between VSMC cultured in the presence or absence of cycloheximide was much greater at 3 and 5 hrs than at 1 hr. This suggests that cycloheximide either increases the half-life of the SM-20 mRNA or prolongs SM-20 transcription.

PDGF Response

As seen in figure 10B, after the addition of PDGF (10 ng/ml BB) SM-20 levels increased at 30 min, peaked at 60-90 min, and then returned to baseline. PDGF and serum thus produced similar patterns of SM-20 mRNA regulation. This is consistent with previous studies demonstrating that PDGF is the major VSMC growth agonist in serum.

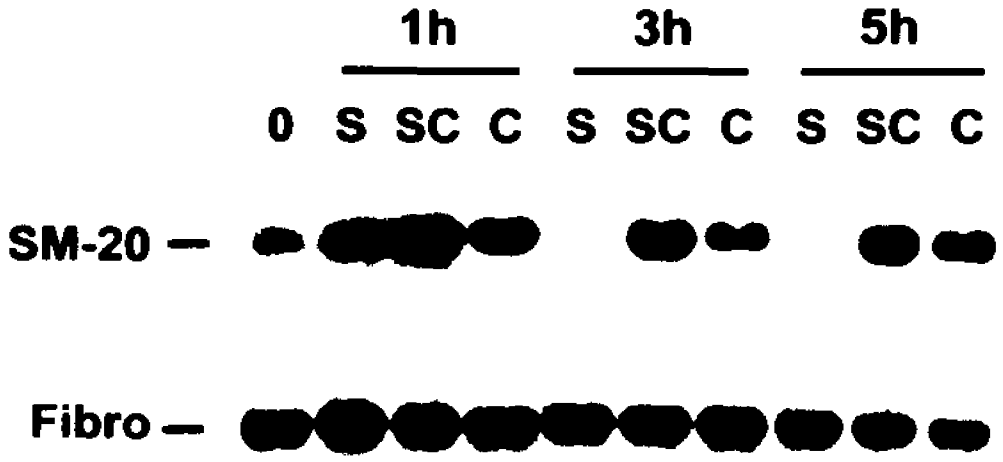


Figure 9. Cycloheximide effects on SM-20 mRNA levels.

Quiescent VSMC (time 0) were treated with serum (S; final concentration 10%), cycloheximide (C; 10μg/ml), or both (SC) and total RNA was collected at the times indicated. Analysis of SM-20 mRNA levels was performed as in figure 8.

Angiotensin II

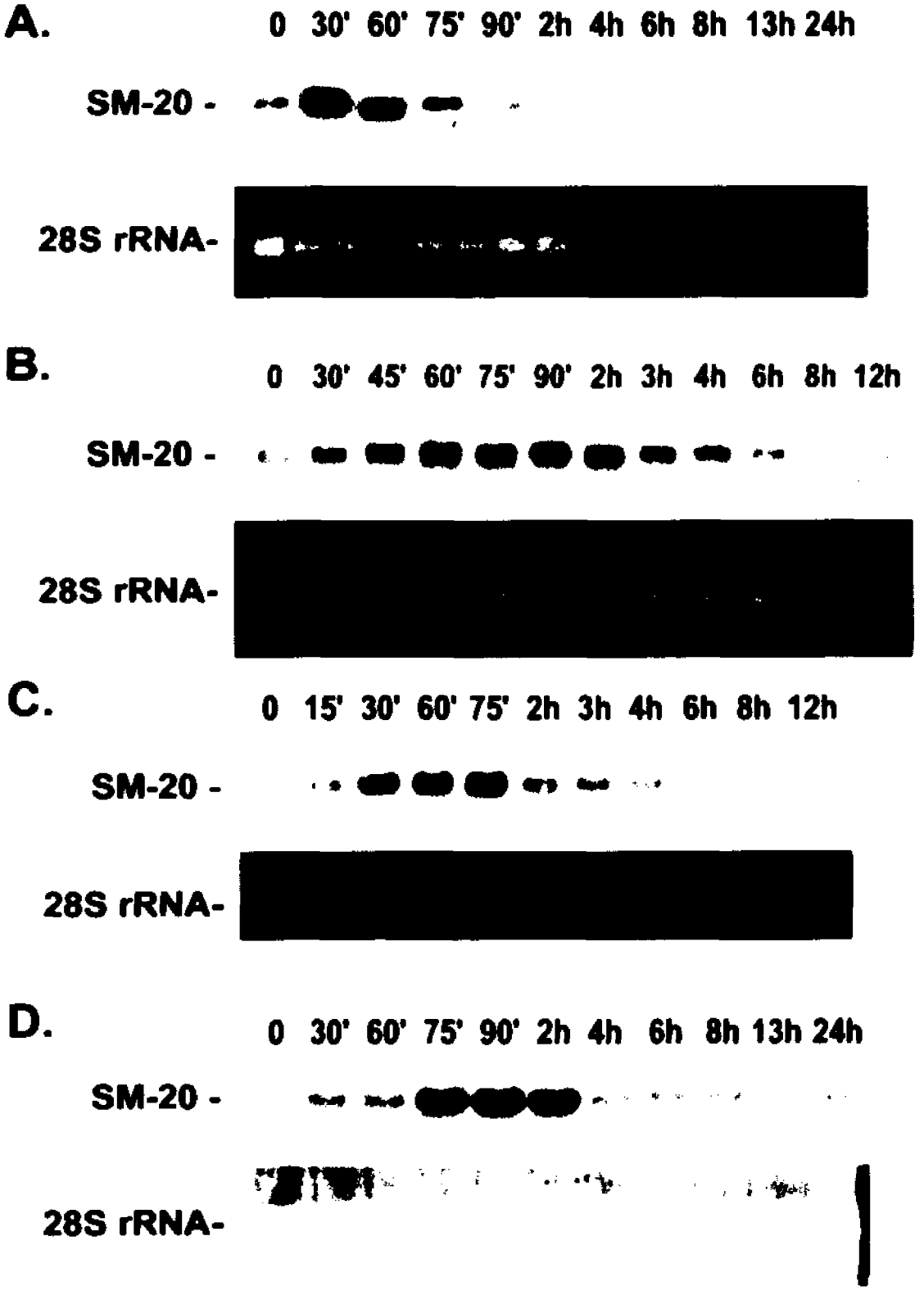
Angiotensin II (Ang II) is an octapeptide present in plasma that is generated by sequential proteolytic cleavages of its less active precursor, angiotensinogen. Ang II is a potent VSMC contractile agonist and a major determinant of blood pressure. Its cell surface receptor, AT1, is a member of the G-protein linked 7 transmembrane-spanning domain receptor family (Murphy et al, 1991). Stimulation of the Ang II receptor (Lyall et al , 1992) activates many of the same gene regulatory intracellular signaling pathways that are activated by PDGF. These include the tyrosine phosphorylation of PLC (Marrero, et al, 1994) and the subsequent stimulation of protein kinase C and the mobilization of intracellular Ca^{+2}. In VSMC culture, Ang II causes cellular hypertrophy, characterized by an increase in cell mass and cell size, but not cell number (Geisterfer et al, 1988; Berk et al, 1989). The regulation of SM-20 mRNA by Ang II is shown in figure 10A. Ang II (10^{-6}M) caused an increase in SM-20 mRNA levels, peaking at 30 min, and then a return to baseline by 2 hrs.

SM-20 mRNA thus displays a similar pattern of regulation during both hypertrophic and hyperplastic growth. Most of the growth factor-responsive genes studied in VSMC also show identical regulation under these conditions (Taubman et al., 1989; Naftilan et. al, 1990; Taubman et al, 1993b; Rothman et al, 1994). Notable exceptions are the genes *JE* and *fos*B, which are only induced during hyperplasia and hypertrophy, respectively (Taubman et al, 1992; Rothman et., al., 1994).

Figure 10. Regulation of SM-20 mRNA by growth and vasoactive agonists.

Quiescent VSMC (time 0) were treated with A) Ang II (1μM), B) PDGF (10ng/ml recombinant BB dimer), C) Isoproterenol (1μM), or D) forskolin (2μM). Total RNA was collected at the times indicated, and analyzed as in figure 8. The ethidium bromide staining of the 28S rRNA, which confirmed the equal loading of RNA samples, is indicated.

Figure 10


A.
0 30' 60' 75' 90' 2h 4h 6h 8h 13h 24h
SM-20 -
28S rRNA-
B.
0 30' 45' 60' 75' 90' 2h 3h 4h 6h 8h 12h
SM-20 -
28S rRNA-
C.
0 15' 30' 60' 75' 2h 3h 4h 6h 8h 12h
SM-20 -
28S rRNA-
D.
0 30' 60' 75' 90' 2h 4h 6h 8h 13h 24h
SM-20 -
28S rRNA-

cAMP effects

One of the regulatory pathways activated by PDGF and serum is the induction of cAMP levels. Increased cAMP has been shown to block the proliferation of VSMC induced by PDGF (Loesberg et al., 1985) as well as serum (Assender et al, 1992). A potential mechanism for this inhibition was demonstrated by Graves et al (1993), who reported that cAMP-dependent protein kinase (protein kinase A) inhibited the activation of mitogen-activated protein kinase (MAPK) and MAPK kinase by PDGF-BB. cAMP increases also lead to VSMC vasodilation, unlike PDGF, Ang II, and serum.

To examine the effect of elevated cAMP levels on SM-20 mRNA, VSMC were treated with isoproterenol. Isoproterenol stimulates β-adrenergic receptors, leading to the activation of membrane-bound adenylyl cyclase via coupling through GTP-binding proteins. Following the treatment of quiescent VSMC with 1μM isoproterenol, SM-20 mRNA levels increased by 30 min, peaked at 75 min and then returned to baseline by 3 hrs (figure 10C). Forskolin is a direct and specific activator of adenylyl cyclase. Stimulation of quiescent VSMC with 2 μM forskolin increased SM-20 mRNA between 75 min and 2hrs, after which levels returned to baseline (figure 10D). These results demonstrate that raising intracellular cAMP is sufficient to increase SM-20 mRNA.

In conclusion, the response of SM-20 to serum, PDGF, Ang II, and cAMP agonists demonstrates that the induction of SM-20 is common to agents which have both positive and negative effects on VSMC growth (including proliferation or hypertrophy).

Tissue distribution

The immediate-early gene family contains both tissue-specific as well as ubiquitously expressed members. Examples of the former include IL-2 and its receptor in T-cells, whereas the proto-oncogenes c-*fos*, c-*myc*, and c-*jun* have been found in all cell types examined to date. Mitogenic stimulation of VSMC and fibroblasts has previously been associated with induction of the same set of genes (Kindy and Sonenshein,1986; Gadeau et al, 1991; Marmur et al, 1992). To determine whether the SM-20 induction is shared by fibroblasts, BALB/3T3 and rat 6 fibroblasts (Hsiao et al, 1986) were cultured under conditions in which other immediate early genes are expressed in fibroblasts and under which SM-20 mRNA is also readily detectable. Northern blot analysis of these cultures revealed no detectable SM-20 mRNA (figure 11A).

The induction of SM-20 thus represents a difference in the immediate-early gene response of cultured VSMC and fibroblasts. The expression of SM-20 may thus serve as a marker for smooth muscle cells even after modulation to a fibroblast-like phenotype. Much of the knowledge regarding VSMC growth has been inferred from studies in cultured fibroblasts. The specific regulation of SM-20 in VSMC, however, suggests that an understanding of VSMC growth may not be simply derived by extrapolation from fibroblasts.

To further characterize the tissue specificity of SM-20 expression, total RNA was collected from tissues and organs of adult male Sprague-Dawley rats (figure 11B). Northern blot analysis of these samples revealed a preponderance of SM-20 mRNA in cardiac, skeletal, and smooth muscle (including esophagus, small bowel, aorta,

and stomach) as well as neuronal cells such as PC12 (Greene and Tischler, 1976) and the brain. Expression was also detected in the tail and kidney. Very low levels were seen in the spleen, liver, lung, and testes. Because the spleen and liver are rich in blood vessels, it is unlikely that the vasculature of the tissues exhibiting high levels of SM-20 mRNA contributed substantially to these levels.

These results indicate that SM-20 is restricted in its expression to specific cell types.

Figure 11. Tissue specificity of SM-20 mRNA expression.

A) Northern Blot analysis of SM-20 expression in NIH 3T3 fibroblasts (lanes 1-7) and rat 6 fibroblasts (lanes 8-11). 48 hrs after the last medium change, the cells reached 80% confluence (lanes 1 and 8), and were then treated for 1hr (lanes 2-4 and 9-11), or 3 hrs (lanes 5-7) with fresh 10% calf serum (lanes 2 and 5), fresh 10% calf serum + 10 µg/ml cycloheximide (lanes 3 and 6), or with 10 µg/ml cycloheximide alone (lanes 4 and 7). As a positive control, each blot contained a lane with 10 µg of total RNA isolated from VSMC treated with 10% calf serum for 1 hr ("VSMC"). To control for possible species specific differences in mRNA sequence accounting for the lack of detectable hybridization to mouse 3T3 cells, 10 µg of total RNA collected from mouse heart ("Heart") was also included. The RNA blot analysis was performed as in figure 8. The integrity of the RNA used for analysis was confirmed by ethidium bromide staining of the 28S (shown above) and 18S rRNA.

B) Total RNA was isolated from the tissues of adult male Sprague-Dawley rats. PC12 cells were grown in 10% horse serum, 5% fetal calf serum. 10 µg of total RNA was loaded per lane and analyzed as in figure 8. Equal loading of lanes was confirmed by ethidium bromide staining of the 28S (shown above) and 18S rRNA.

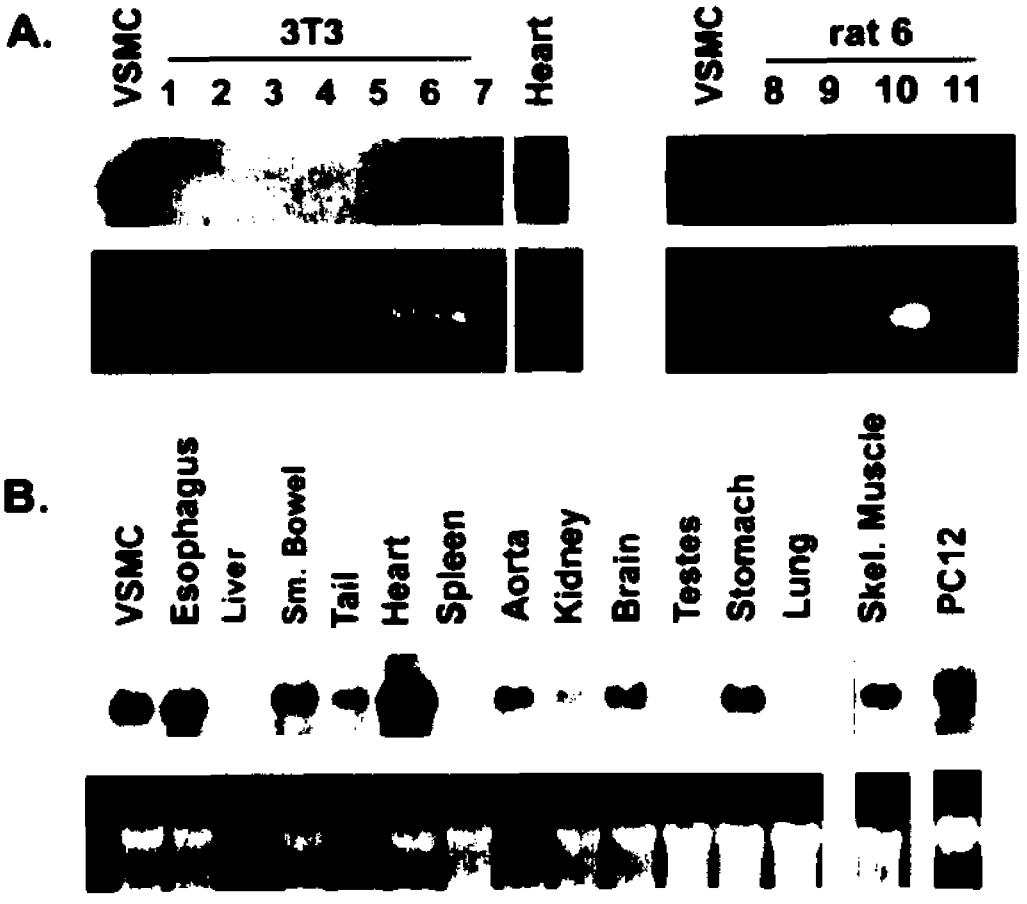

A.
VSMC
3T3
1 2 3 4 5 6 7
Heart
VSMC
rat 6
8 9 10 11
B.
VSMC
Esophagus
Liver
Sm. Bowel
Tail
Heart
Spleen
Aorta
Kidney
Brain
Testes
Stomach
Lung
Skel. Muscle
PC12

Figure 11

Chapter 5: Analysis of SM-20 protein

Analysis of the SM-20 combined sequence revealed an open reading frame encoding a 355 amino acid protein. The peptide sequence showed significant homology only to the putative peptide sequence of an uncharacterized partial cDNA cloned from C. elegans. It was therefore imperative to establish that the SM-20 open reading encoded a protein expressed in VSMC. Toward this end, antibodies which specifically recognized the SM-20 putative peptide sequence were needed. A 28 kD immunogen was prepared by expressing SM-20 amino acid residues 126-355 (see figure 3) in E. coli as part of a fusion peptide whose synthesis was induced by IPTG (figure 12).

A bacterial extract highly enriched for the SM-20 immunogen was prepared (see Materials and Methods), and subjected to denaturing polyacrylamide gel electrophoresis. Coomassie blue staining of the gel revealed that the expressed peptide migrated at the expected molecular weight (28 kD), and accounted for nearly all of the protein present in the enriched extract (figure 13). To prepare protein for immunization, a gel fraction containing approximately 100µg of the peptide was excised. This fraction was mixed with an aluminum-based adjuvant, and used for subcutaneous injections of a rabbit. Two weeks after the injection, the serum was collected and the anti-SM-20 antibodies were affinity purified.

Enzyme-linked immunosorbent assays (ELISA) were used to titre the antisera. One µg of the enriched bacterial lysate was adsorbed onto microtitre wells. Serial dilutions of the pre-immune and the immune serum were added to the wells. Antibody binding

was detected with an alkaline phosphatase-conjugated secondary antibody. Preimmune serum dilutions greater than 1:4 produced little background color. In contrast, the immune serum produced strong color reactions even at 1:200 dilutions. This suggests that antisera had successfully been raised against the bacterially expressed SM-20 sequence and provided an approximation for the concentration of antisera to use in subsequent experiments.

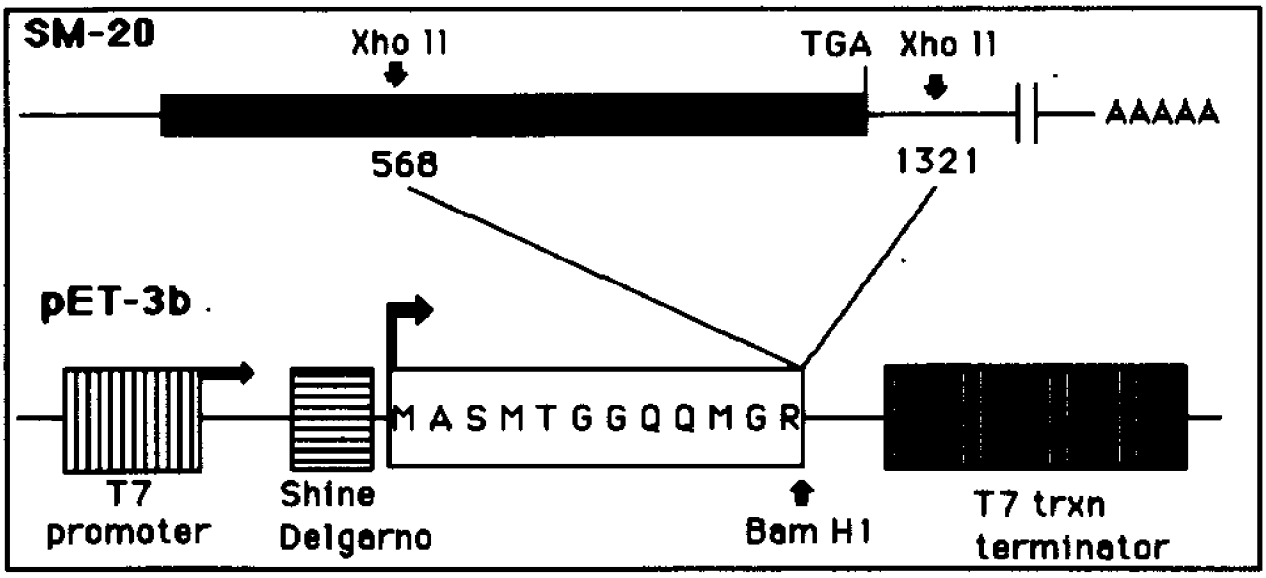


Figure 12. Construction of pET-SM-20 vector for production of SM-20 immunogen. cDNA clone SM-20/2 was digested with Xho II, and the resulting fragment extending from base 568 to 1321 was ligated in frame into the pet-3b (Novagen) bacterial expression vector. DNA sequence analysis confirmed that the resulting vector encoded a fusion protein consisting of the first 11 amino acids of the phage T7 gene *10* capsid protein, an arginine residue, and residues 126 to 355 of the SM-20 putative peptide sequence. Transcription of this sequence is under the control of the phage T7 RNA polymerase promoter. This vector was transformed into the E. coli strain BL21 which contains the T7 polymerase gene under the control of the lac Z promoter. The addition of 0.5mM IPTG to log-phase cultures of the transformants induced high levels of the fusion peptide.

➡ transcription start site ↱ translation start site

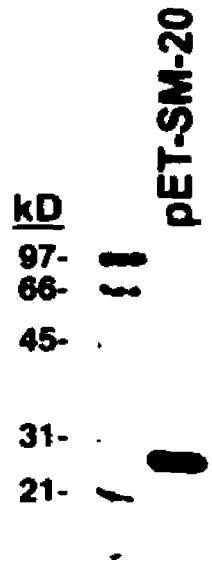


Figure 13. Analysis of the SM-20-enriched bacterial lysate by Coomassie blue staining.

BL21 E. coli were transformed with the pET-SM-20 vector (see figure 12), and a lysate enriched for the SM-20 fusion peptide was prepared (see materials and methods). 7μg of this lysate was size fractionated by electrophoresis on a SDS-polyacrylamide (12%) gel, and then stained with Coomassie Brilliant blue. A protein species is observed which migrates with an apparent molecular weight that is consistent with the expected size of 28kD. This protein accounts for nearly all of the protein observed. The positions of the prestained molecular weight standards are also seen.

Recognition of SM-20 protein in VSMC extracts

To determine the size and number of VSMC protein species detected by the antisera, immunoblotting of SDS- polyacrylamide gels was performed. Quiescent VSMC were scraped into Laemmli buffer (2%SDS, 100 mM DTT, 50 mM Tris, pH 6.8, 10% glycerol) to prepare a total cellular extract. Twenty Five μg of this extract was loaded into lanes of a denaturing polyacrylamide gel. Thirty ng of the enriched bacterial lysate was also loaded onto the gel as a positive control. Following electrophoresis and transfer to nitrocellulose, the filter was cut into sections and incubated with either the pre-immune or the immune sera. Bound antibody was detected using an enhanced chemiluminescence kit. As seen in figure 14, under the assay conditions the preimmune serum did not detect any protein species in either the BL21 or VSMC lysate. In contrast, the immune serum recognized both the 28kD immunogen in the bacterial extract, as well as one major protein in the VSMC lysate that migrated with an apparent molecular weight of approximately 40 kD. This is consistent with the predicted molecular weight of the putative peptide sequence. These results suggest that the ORF identified in the SM-20 "combined sequence" (derived from the genomic DNA and cDNA clones) does encode a protein produced in VSMC. Consistent with the lack of an identifiable hydrophobic signal sequence in the putative SM-20 peptide, this protein species was not detected in a TCA precipitate derived from the conditioned medium of a 100mm culture plate.

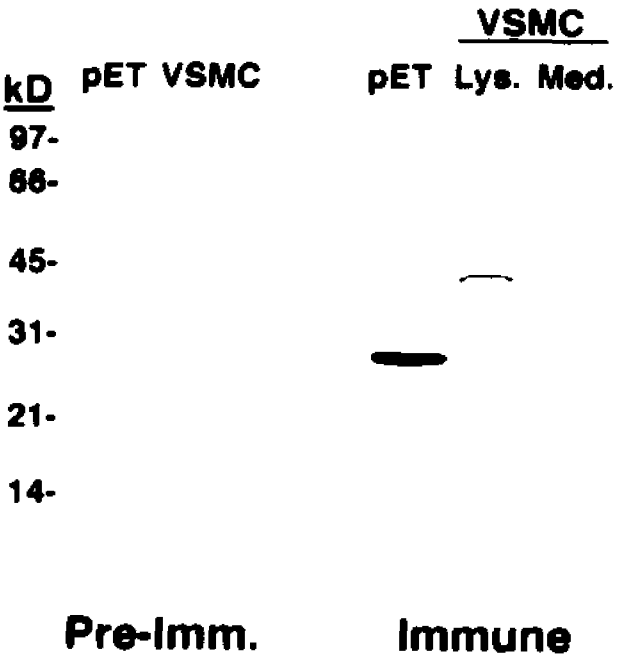


Figure 14. Western blot analysis of SM-20 protein.

Protein samples consisting of 30 ng of a bacterial extract enriched for the SM-20 fusion peptide (pET), 25 µg of total VSMC protein (VSMC), or a total TCA precipitate from 6ml of VSMC culture medium (Med.) were prepared in Laemmli buffer (2% SDS, 100 mM DTT, 10% glycerol, 100 mM Tris, pH 6.8). Following size fractionation by electrophoresis on a SDS-polyacrylamide (10%) gel and transfer to nitrocellulose, filters were incubated with either an affinity purified "pre-immune" or "immune" serum. A horseradish peroxidase-conjugated secondary antibody was used in conjunction with an enhanced chemiluminescence kit to identify the proteins detected by the primary antibody. Biotinylated molecular weight markers were included. The positions of the markers were visualized by incubation with avidin-conjugated horseradish peroxidase, followed by enhanced chemiluminescence.

Analysis of SM-20 protein regulation *in vitro*

SM-20 mRNA levels are regulated in cultured VSMC in response to serum stimulation, and thus a corresponding regulation of the protein would be expected. To characterize the SM-20 protein response, Western blot analysis was performed. As seen in figure 15A, SM-20 antigen was detected in quiescent rat VSMC. Following the addition of fresh 10% calf serum, the SM-20 levels increased, peaking at 2 hrs, and then decreased towards baseline. This pattern of regulation is consistent with the SM-20 mRNA response to serum, where the peak induction was seen at approximately 1 hr. SM-20 antisera also detected a protein species in quiescent human VSMC which migrated at approximately the same molecular weight as the rat SM-20. In an initial examination, this putative human SM-20 protein was increased at 3hrs of serum stimulation (figure 15B). These results support the conclusion that the protein species detected by immunoblotting is derived from the SM-20 ORF, and that SM-20 protein levels are regulated in VSMC. Human and rat VSMC appear to express antigenically related SM-20 proteins.

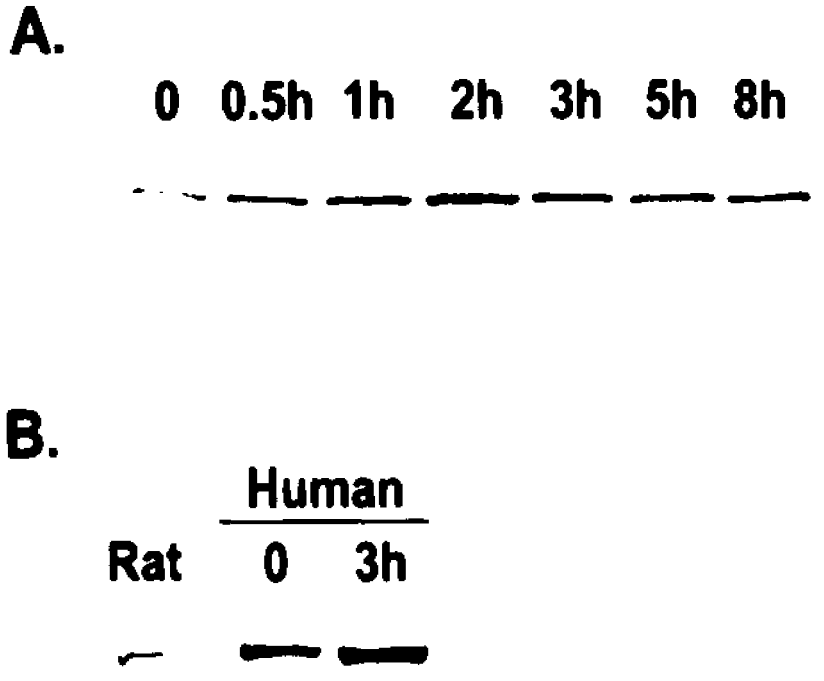


Figure 15. Western blot analysis of SM-20 protein regulation by serum
A) Rat VSMC: Quiescent rat VSMC (time 0) were treated with fresh 10% calf serum, and total cellular protein was collected in Laemmli buffer at the times indicated. 25µg of each protein sample was loaded onto a SDS-polyacrylamide (10%) gel. Immunoblot analysis was performed as described in figure 14.
B) Human VSMC: Total cellular protein was collected from quiescent (time 0; incubated in serum-free medium for 48hrs) and 10% fetal calf serum-stimulated (3hrs) human VSMC. 25µg of each human VSMC protein sample and 25µg of total rat VSMC protein (collected from quiescent cells) were loaded onto lanes of a SDS-polyacrylamide (10%) gel and analyzed by immunoblotting as described in figure 14.

Subcellular localization

To characterize the subcellular location of SM-20 protein, immunocytochemistry was performed on cultured VSMC. The pre-immune serum did not stain the VSMC (figure 16A). In contrast, the post-immunization antisera recognized an antigen with a peri-nuclear pattern of distribution (figure 16B). Immunoreactive antigen was not detected within the nucleus, plasma membrane, or extracellular space. This same pattern of staining was observed in cultures of human aortic smooth muscle (figure 16C and D). These results raise the possibility that SM-20 is associated with the outer nuclear membrane and endoplasmic reticulum and suggest that the antigen is conserved between the human and rat.

Antisera specificity

The specificity of the affinity-purified antisera for the SM-20 peptide sequence was verified by competitive inhibition of the immunocytochemical staining. Extracts were made from bacteria transformed with either the pET vector containing the SM-20 insert, or the pET vector alone. The proteins of these extracts were then adsorbed onto nitrocellulose filters. As seen in figure 17A, preincubation of the antiserum on filters containing the SM-20 immunogen blocked the cellular staining. In contrast, preincubation with the bacterial extract lacking the SM-20 sequence failed to inhibit binding (figure 17B). This indicates that the VSMC epitopes recognized by the antisera are present within the bacterially expressed SM-20 peptide.

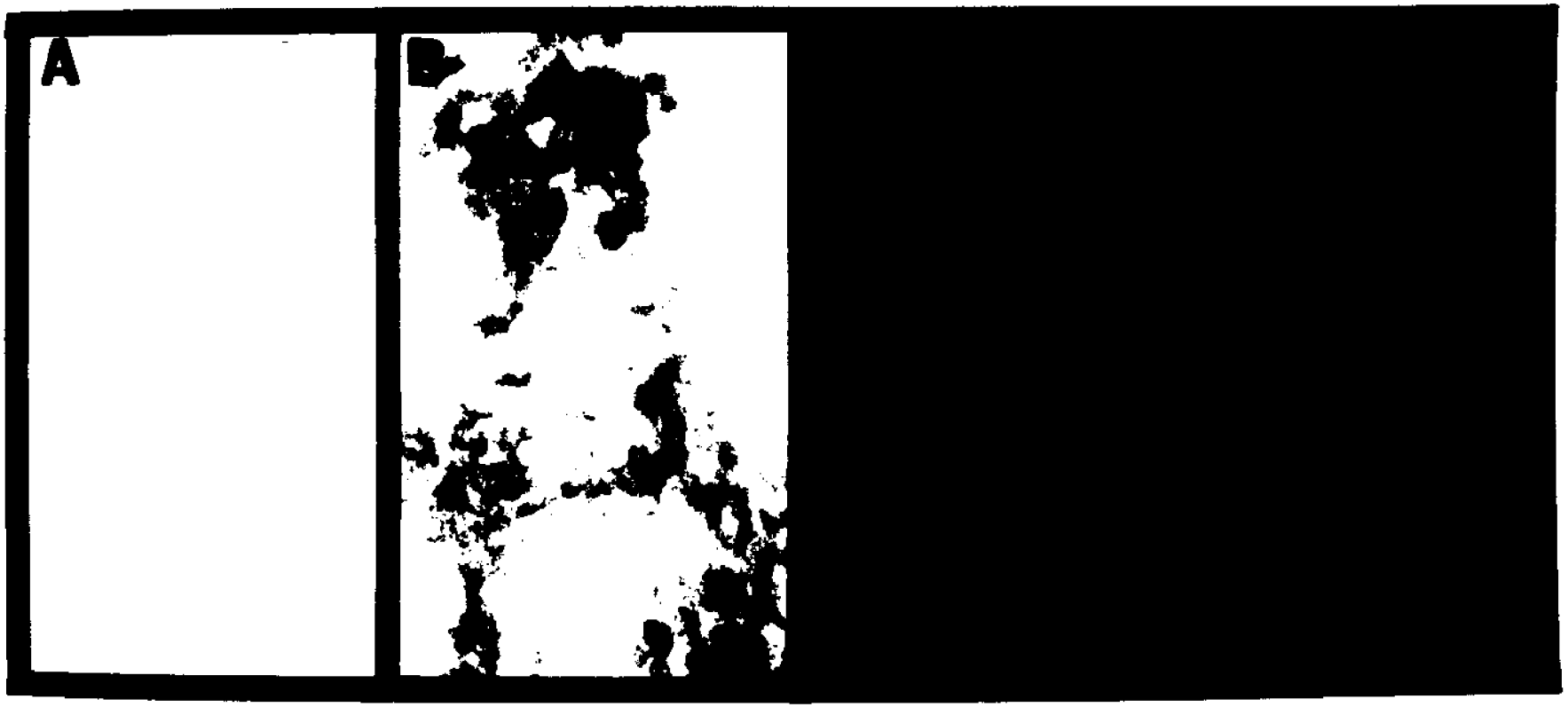


Figure 16. Cellular localization of SM-20 protein in VSMC

Immunohistochemical analysis of SM-20 immunoreactive protein in rat (A,B) and human (C,D) VSMC. VSMC were grown to 80% confluence and then made quiescent by incubation in 0.3 % calf serum (rat) or serum-free medium (human) for 48 hrs. The cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide. The affinity purified "pre-immune" (A,C) or "immune" (B,D) antiserum was added to each well. Sequential incubations with a biotinylated secondary antibody, avidin-horseradish peroxidase complex, and AEC substrate were used to detect the primary antibody.

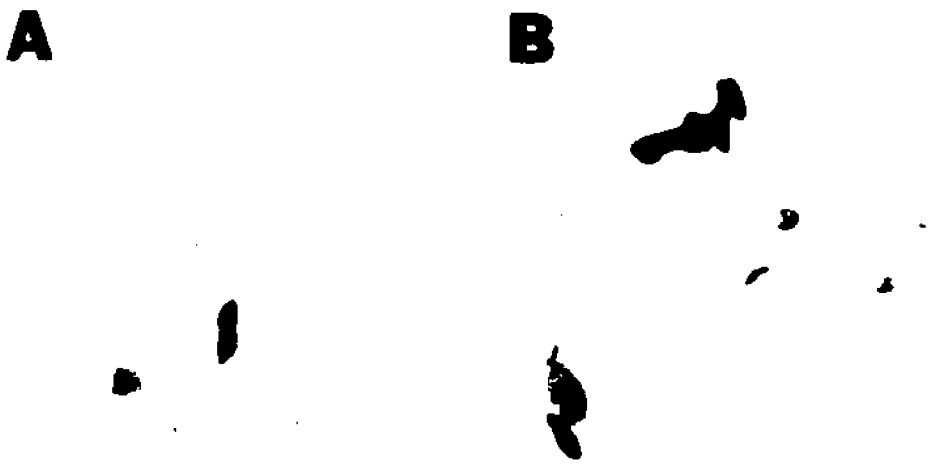


Figure 17. Inhibition of SM-20 antisera binding with SM-20 peptide.

VSMC were stained as described in figure 16. Prior to immunostaining, SM-20 antisera was pre-incubated with nitrocellulose filters adsorbed to bacterial extracts which either contained (A) or lacked (B) the SM-20 fusion peptide. The antiserum was removed from the filters and used for immunostaining.

Analysis of SM-20 expression in the vessel wall

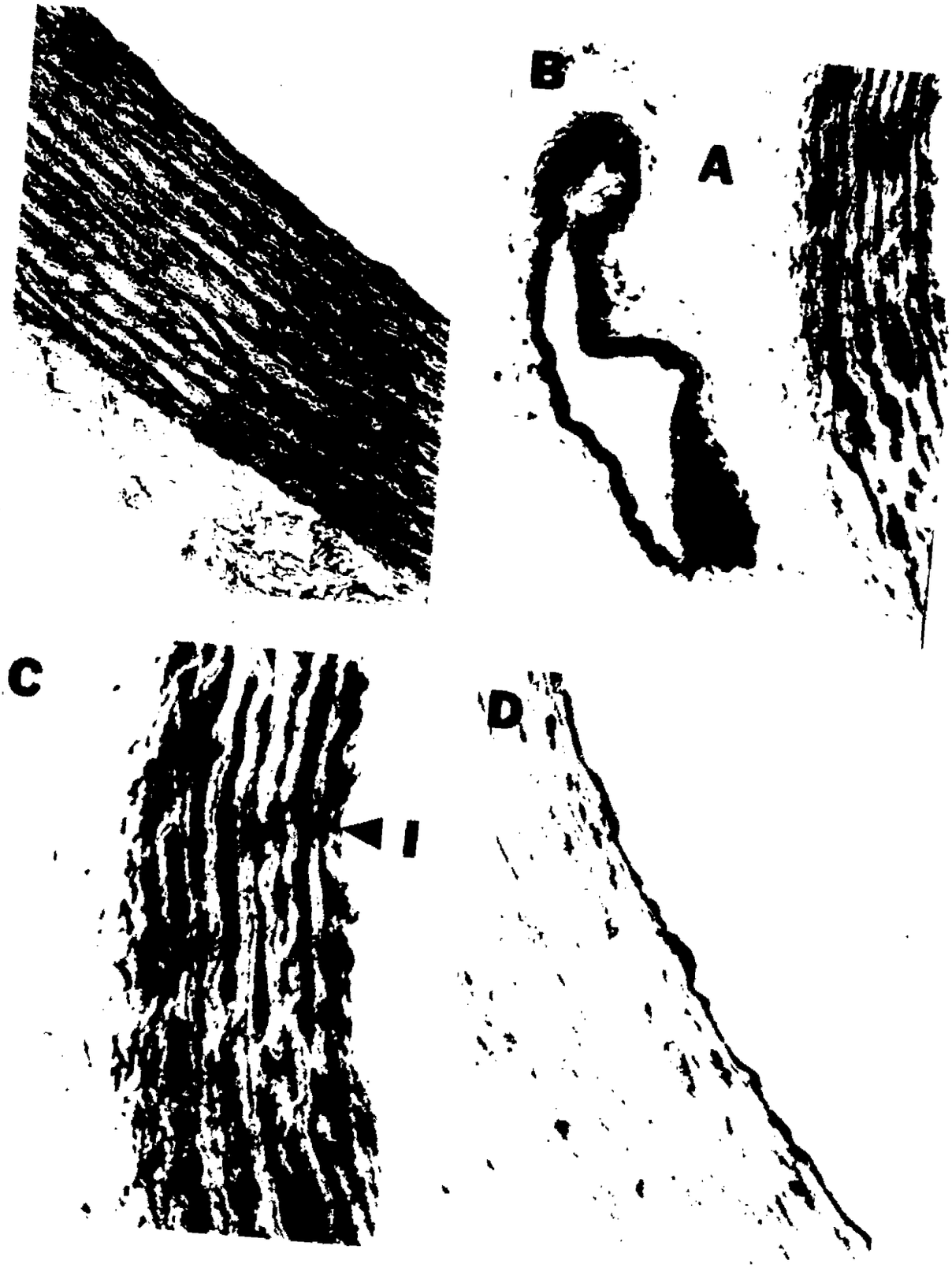
Normal rat aorta

Northern blot analysis demonstrated that SM-20 mRNA was expressed in the aorta of adult rat (figure 11B). To establish the presence and cellular distribution of SM-20 protein in the vessel wall, immunohistochemistry was performed. Aortic cross sections were prepared from paraformaldehyde-perfused Sprague-Dawley rats and incubated with either the pre-immune or the immune antiserum. The pre-immune serum did not recognize vessel wall antigens (figure 18A), whereas the immune serum revealed SM-20 protein within the smooth muscle in the tunica media of the aorta and of an arteriole (figure 18B). Significantly, SM-20 protein was not detected in the adventitial fibroblasts. This pattern of expression is consistent with the analysis of SM-20 mRNA expression in cultured VSMC and fibroblasts (see figure 11A). Staining was not observed in the tunica intima (figure 18B and at higher magnification in 18C), suggesting that SM-20 protein is not expressed by the endothelium. The integrity of the endothelial cell layer in these tissue sections was confirmed by immunostaining with antisera against Von Willebrand factor, an endothelial cell-specific marker (figure 18D). SM-20 protein thus appeared to be restricted to the VSMC of the vessel wall.

Figure 18. Analysis of SM-20 protein expression in the normal rat vessel wall.

The aortas of adult male Sprague-Dawley rats were fixed in 4% paraformaldehyde and frozen in liquid nitrogen. 8 micron sections were placed onto slides, endogenous peroxidase activity was quenched using periodic acid (Peroxo-block, Zymed), and then incubated with the "pre-immune" (A) or "immune"(B,C) affinity-purified SM-20 antiserum, or an anti-Von Willebrand factor antiserum (D). Sequential incubations with a biotinylated secondary antibody, avidin-horseradish peroxidase conjugate, and the DAB substrate were used to detect the primary antibody. C) is a higher magnification of the aorta stained in B), allowing closer examination of the intima (I). SM-20 immunoreactive protein was detected in VSMC in the tunica media (M) of the aorta and of an arteriole (BV). SM-20 was not detected in the fibroblasts of the adventitia (A).

Figure 18



Injured rat aorta

As discussed in the introduction, the smooth muscle cells that comprise the tunica media of an intact adult possess a contractile phenotype and are mostly quiescent. The neointimal smooth muscle cells of the atherosclerotic plaque, however, are in a synthetic (proliferative) phenotype. To determine whether SM-20 protein levels differ between these cell types, the aortas of adult rats were injured by balloon angioplasty. Two weeks after this injury, a neointima comprised of synthetic smooth muscle cells had formed. Immunohistochemistry using the SM-20 antisera revealed that the level of SM-20 protein was markedly elevated in the neointimal smooth muscle relative to the medial smooth muscle (figure 19B). This suggests that SM-20 is more highly expressed in proliferative than contractile VSMC.

The regulation of SM-20 mRNA levels in the vessel wall 2 wks after balloon injury was also examined by *in situ* hybridization. Sense and anti-sense riboprobes were synthesized from the 450 nucleotides at the 3' end of cDNA clone SM-20/2. The sense probe displayed a uniform, low level of background hybridization (figure 19C). In contrast, the antisense probe gave a strong signal in the aortic media and a markedly higher signal in the neointima (figure 19D). The SM-20 mRNA expression pattern thus reflects the protein expression pattern revealed by immunohistochemistry.

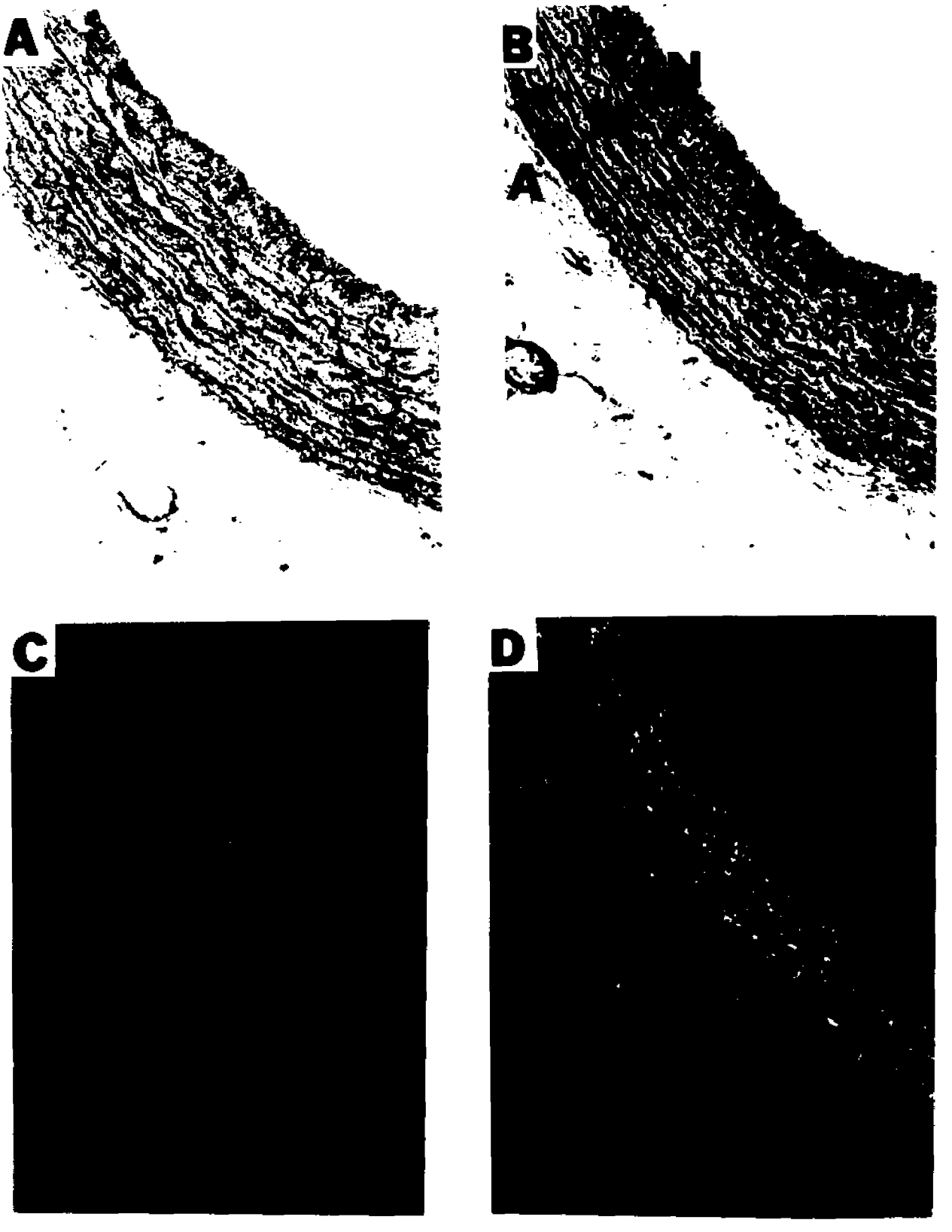
Figure 19. Analysis of SM-20 expression in neointimal and medial VSMC.

The aortas of adult male Sprague-Dawley rats were injured by balloon angioplasty. After 2 weeks, aortas were isolated, fixed in 4% paraformaldehyde, frozen in OCT, and 8 micron sections were prepared. The neointimal (N), medial (M), and adventitial (A) regions are indicated.

A,B) Immunohistochemical analysis: Sections were incubated with either preimmune (A) or immune (B) SM-20 antisera. Detection of antibody binding was performed as in figure 18 using AEC substrate.

C,D) *In situ* hybridization analysis: Sense (C) and antisense (D) ^{35}S-labeled riboprobes were generated from the 450 bases at the terminus of the 3' untranslated region of cDNA clone SM-20/2 (see figure 2A).

Figure 19



Human atherosclerotic coronary arteries

In collaboration with Dr. John Fallon, the pattern of SM-20 protein expression in adult human atherosclerotic coronary arteries was examined by immunohistochemistry. As seen in figure 20A and B, the level of immunoreactive SM-20 protein was markedly elevated in the VSMC of the neointimal plaque relative to the medial smooth muscle. SM-20 protein was not detected in the endothelium which lined the lumenal surface of the plaque (figure 20C), or in the adventitial fibroblasts (figure 20A). The restricted pattern of expression and predilection for the neointimal VSMC mimic that described above for the rat aorta.

SM-20 thus appears to be differentially regulated *in vivo* in proliferative and contractile smooth muscle. These results, in combination with the analysis of the SM-20 response to growth factors in VSMC culture, support the hypothesis that the regulation of SM-20 levels may be important for VSMC growth.

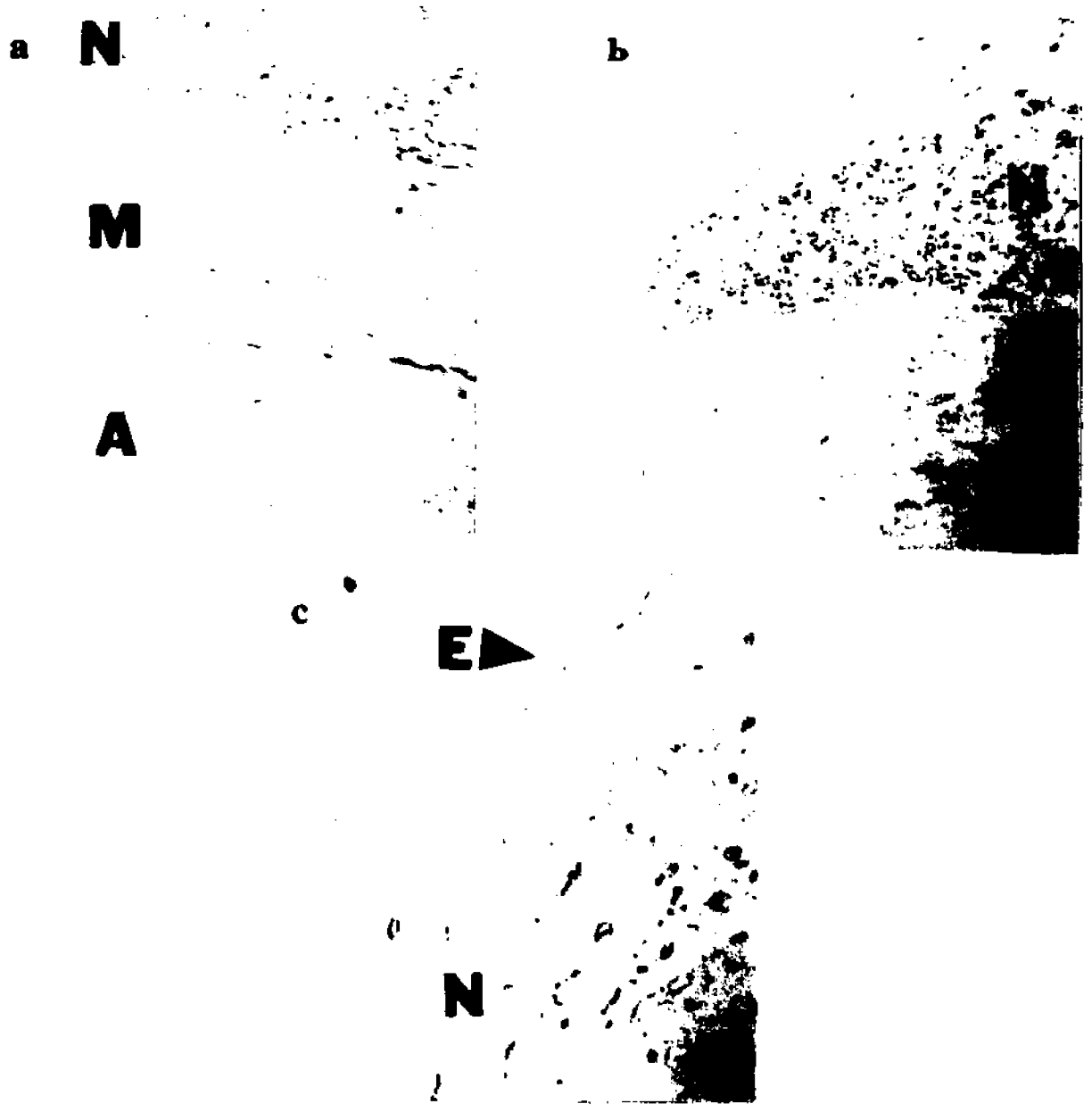


Figure 20. Immunohistochemical analysis of SM-20 protein expression in human atherosclerotic coronary arteries.

Sections of human coronary arteries were obtained from autopsy specimens provided by Dr. John Fallon. The regions of the arterial media (M), neointima (N), endothelium (E), and adventitia (A) are indicated. SM-20 immunoreactive protein was detected in the medial and neointimal VSMC but not in the adventitial fibroblasts. The neointimal VSMC expressed markedly higher levels of SM-20 than medial cells, as seen in a), b), and c). Staining was not observed in endothelium (c).

Chapter 6: Analysis of SM-20 protein expression in skeletal muscle

Striated muscle of the esophagus

Northern blot analysis indicated that skeletal muscle expresses high levels of SM-20 mRNA (figure 11B). To examine the expression of SM-20 protein in skeletal muscle *in vivo*, immunohistochemistry was performed on sections of the lower esophagus (which contains skeletal muscle) using the SM-20 antisera. High levels of SM-20 protein were detected in the striated muscle (figure 21A) by the immune serum, whereas a very low signal was produced by the pre-immune serum (figure 21B). SM-20 protein was not detected in the cells (most likely fibroblasts) of the connective tissue (fascia) that surrounds the muscle fibers. This pattern of staining is consistent with that seen in the arterial wall, i.e. SM-20 protein was detected in the muscle cells but not in the adventitial fibroblasts.

Regulation during differentiation *in vitro*.

C2C12 is a skeletal myoblast cell line (reported by Yaffe and Saxel, 1977, and subsequently characterized by H. Blau and colleagues, Stanford University) that can be maintained and propagated in the undifferentiated state when high levels of growth factors (e.g.15% fetal calf serum) are present in the culture medium. These cells can be induced to differentiate into fused myotubes by switching the culture medium to 3% horse serum when the cells approach confluence. At confluence, some of the cells will differentiate even in the presence of growth factors.

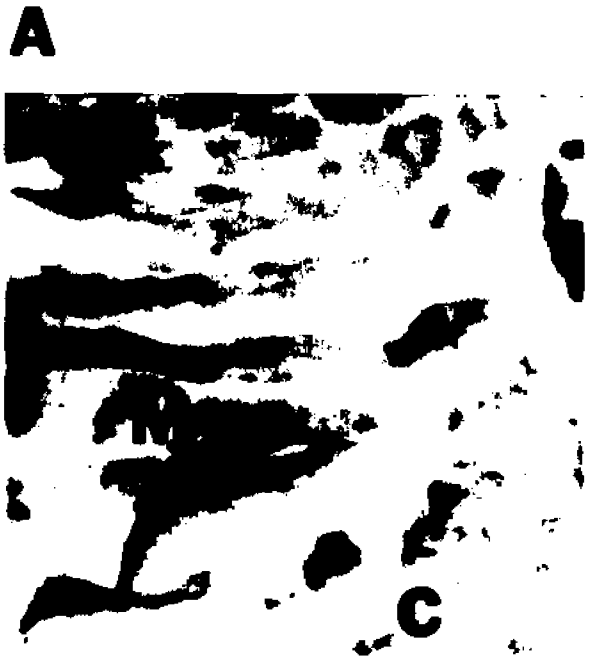


Figure 21. Immunohistochemical detection of SM-20 protein in skeletal muscle of the esophagus.

The lower esophagus of an adult male Sprague-Dawley rat was fixed in 4% paraformaldehyde and frozen in OCT. Sections were incubated with either the SM-20 immune (A) or preimmune (B) affinity-purified antiserum. Detection of the primary antibody was performed as in figure 18 using AEC substrate. Striated muscle fibers (M) and connective tissue (C) are indicated.

To characterize the size and number of protein species detected by the SM-20 antisera in C2C12 cultures, Western blot analysis was performed. C2C12 cells were grown to confluence in 15% fetal calf serum and the total cellular protein was collected in Laemmli buffer. Immunoblotting with the affinity purified antisera detected a single major protein in C2C12 extracts which migrated at the same position as the SM-20 protein in VSMC culture (figure 22).

The expression of SM-20 during C2C12 differentiation was examined by immunocytochemistry. Proliferating, undifferentiated C2C12 cells contained low levels of SM-20 immunoreactive protein (figure 23A). However, following differentiation in 3% horse serum, high levels of SM-20 antigen were observed in the fused myotubes (figure 23 C and D). Interestingly, SM-20 was not detected in the cells which failed to become incorporated into fused myotubes (figure 23 C and D). When cultures of C2C12 cells reach confluence, even in the presence of 15% fetal calf serum, some of the cells begin to fuse and undergo myogenic differentiation. SM-20 protein was detected in the fused cells of these cultures (figure 23B). These results indicate that the expression of SM-20 in these cultures is correlated with the differentiated phenotype, and is not simply a response to horse serum.

The striking induction of SM-20 protein levels in C2C12 myotubes suggests that SM-20 is involved in cellular functions specific to the differentiated state. This may include a significant role in the differentiation process.

Figure 22. Western blot analysis of SM-20 antigen in C2C12 culture.

C2C12 myoblasts were grown to confluence in 15% fetal calf serum and total cellular protein was collected in Laemmli buffer. 25 µg of protein ("C2C12") was loaded onto a SDS-polyacrylamide (10%) gel and size fractionated. 25µg of total rat VSMC protein ("VSMC") was included. Immunoblotting was performed as in figure 14.

Figure 23

A

B

C

Figure 23: Immunocytochemical analysis of SM-20 protein levels during C2C12 differentiation.

50% confluent cultures of proliferating C2C12 cells (A) were maintained in 15% fetal calf serum until reaching 80% confluence. Duplicate cultures were then incubated for 4 days in either 3% horse serum to induce differentiation (B, D, E), or 15% fetal calf serum to produce confluence (C). These cultures were then immunostained using either the SM-20 preimmune (E) or immune (A,B,C,D) serum as described in figure 16. D) is a higher magnification of the same culture plate as seen in B). High levels of SM-20 immunoreactive protein were detected in the differentiated myotubes (shaded arrows), but not in the unfused or proliferating myoblasts (open arrows).

Chapter 7: Discussion

This thesis describes the identification and characterization of SM-20, a novel growth factor-responsive gene. SM-20 was isolated by differential screening of a VSMC cDNA library. The nucleotide and deduced amino acid sequence of SM-20 demonstrate significant homology only to an uncharacterized partial cDNA cloned from C. elegans. SM-20 is regulated by growth factors and vasoactive agonists in VSMC culture. The SM-20 protein appears to be localized to the peri-nuclear cytoplasmic region of VSMC. Expression of SM-20 appears to be restricted to the VSMC of the vessel wall and is increased in the VSMC of the neointima. SM-20 is also induced during the differentiation of skeletal myotubes.

Differential screening of a VSMC cDNA library identified the SM-20 cDNA clone as hybridizing with less intensity to mRNA collected from PDGF-treated than from quiescent VSMC. This raised the possibility that SM-20 corresponded to an mRNA that was down-regulated by growth factors. Examination of the SM-20 response to growth factors in greater detail revealed that SM-20 levels were actually increased at 1 hr following stimulation before returning to or below baseline at 3 hrs. This underscores one of the pitfalls of the differential screening strategy, i.e. that the difference between populations of cells at 2 distinct time points may not reflect the pattern of regulation. In a more general sense, this result is instructive in demonstrating the value of collecting ample data before characterization of a natural phenomenon is made.

Lau and Nathans (1985; 1987) as well as Almendral et al (1988) each reported differential screening of approximately 2 X 10^5 recombinant phage plaques with ^{32}P-cDNA probes derived from 3T3 fibroblasts that were quiescent or stimulated with serum and cycloheximide. These groups identified 10 and 82 independent cDNA sequences, respectively. This thesis describes the differential screening of approximately 4 X 10^4 plaques which identified 5 genes regulated in VSMC following the addition of serum alone. The frequency of differentially regulated sequences within the VSMC cDNA library (≈1.25 clones/10^4 plaques) is thus intermediate to that reported previously for 3T3 fibroblasts (0.5-4 clones/10^4 plaques).

SM-20 putative peptide sequence analysis

Screening of cDNA and genomic DNA libraries led to the identification of a single large open reading frame which encoded a peptide with a predicted molecular weight of 39.7 kD. The assignment of the initiating ATG codon is based on several observations. This codon is within a sequence that is predicted to be an efficient site for translation initiation based on the consensus sequence defined by Kozak. RT-PCR demonstrated that an upstream in-frame stop codon, located 81bp from the ATG, was within the SM-20 mRNA (and not part of an intron). The assigned ATG is the first ATG to be in frame with the single large open reading frame. In addition, antisera raised against a 230 amino acid region of this ORF identified a protein in VSMC lysates that migrated with an apparent molecular weight of approximately 40kD, which is consistent with the size of the predicted SM-20 peptide.

The SM-20 protein sequence appears to be highly conserved. Western blot analysis identified a protein expressed in mouse (C2C12) and human (VSMC) cells which migrates at the same apparent molecular weight as the rat SM-20 protein. Immunocytochemical analysis revealed that SM-20 had a similar cellular localization in human and rat VSMC. Immunohistochemistry of human and rat arteries also demonstrated similar patterns of regulation. The putative amino acid sequence of the C. elegans clone M88987 contained high homology (35/50 amino acid identity) to the rat peptide sequence. This high degree of conservation in sequence between two species as evolutionarily distant as the rat and C. elegans may indicate that SM-20 and M88987 perform analogous functions. The C. elegans sequence was derived from one of approximately 1200 cDNA clones isolated as an adjunct to the C. elegans genomic sequencing effort. Thus, only 389 bp was sequenced and the identity of the protein was not determined. It will be of interest to isolate the complete C. elegans cDNA and compare its predicted amino acid sequence to the SM-20 sequence. Significant differences and similarities in these sequences may reveal functionally important domains.

The lack of SM-20 homology to other Genbank sequences provided few clues as to the function of SM-20. The SM-20 peptide does contain one RGD sequence. This motif has been found to mediate the binding of extracellular proteins to integrins (located in the plasma membrane). Because SM-20 protein was detected only intracellularly, the functional significance of this motif is unclear. The extremely basic pI (9.93) may indicate that SM-20 is associated

with membranes, whose surfaces are negatively charged. A consensus sequence for phosphorylation by protein kinase C and for calmodulin-dependent kinase II (CaMK II) are each present within SM-20. These are of particular significance because serum, PDGF, and Ang II activate protein kinase C and mobilize intracellular calcium. Thus in addition to increasing the level of SM-20 in VSMC, these agents may modify the activity of SM-20 by phosphorylation. A change in the molecular weight of SM-20 following serum-stimulation of quiescent vsmc was not observed. However, phoshorylation of only a few residues, such as by PKC and CaMK II as mentioned above, could be detected by the addition of $^{32}PO_4$ to quiescent vsmc before growth factor stimulation. Alternatively, phosphorylation of SM-20 protein by either PKC or CaMK II could be tested *in vitro*.

Implications of induction by cycloheximide

The pattern of SM-20 mRNA regulation was characteristic of the immediate-early gene family; i.e. the mRNA level was increased by cycloheximide and induced by serum even in the presence of cycloheximide. This demonstrates that SM-20 is part of the first set of genes activated following growth factor stimulation. As postulated for other members of the immediate-early gene family, SM-20 may be involved in the regulation of subsequent cellular processes determining the VSMC growth state.

The induction and prolonged elevation of SM-20 mRNA levels in the presence of cycloheximide suggest possible mechanisms by which SM-20 is regulated. A labile protein(s) might act to destabilize

the mRNA or to repress SM-20 transcription. The SM-20 3' untranslated region contains three AUUUA sequences, which have been shown to mediate mRNA destabilization (Shaw and Kamen, 1986; Shyu et al, 1991). Blocking synthesis of the cellular factors that recognize these sequences may thus contribute to the induction and prolonged elevation of SM-20 mRNA by cycloheximide. Linking the SM-20 3' untranslated region to an alternate coding region might result in a cycloheximide-responsive transcript.

Implications of cAMP-responsiveness

The induction of SM-20 by cAMP is unusual among the immediate-early genes in VSMC. For instance, although c-*fos*, *jun*-B, and c-*myc* are cAMP-responsive in other cell types such as fibroblasts, they are not induced by cAMP in VSMC (Taubman et al, 1992; Taubman et al, 1989). Forskolin treatment of VSMC is also not sufficient to induce *JE*, *KC*, tissue factor, or c-*jun* (Taubman et al, 1992, and unpublished observations). This suggests that in VSMC the induction of SM-20 occurs via pathways which are different from these other immediate-early genes.

Based on the large amount of previous work on cAMP-mediated gene induction, the increase in SM-20 mRNA levels by cAMP is likely to be mediated by increased SM-20 transcription (Karin, 1992). Intracellular cAMP binds to the regulatory subunit of protein kinase A (PKA), releasing the catalytic subunit which then translocates to the nucleus. Phosphorylation of transcription factors by PKA increases their gene regulatory activity, probably by increasing the potential of their transcription-activating domains. A

palindromic cis-acting sequence referred to as a cAMP-response element (CRE), mediates the responses to the PKA-inducible transcription factors. The induction of SM-20 by isoproterenol and forskolin suggests that the SM-20 promoter contains a CRE.

The putative SM-20 CRE may be a point of convergence for several of the gene-regulatory intracellular signaling pathways activated by PDGF and serum, including PKA, PKC, and mobilization of intracellular Ca^{+2}. Members of the CRE-binding (CREB) protein family possess a basic domain/ leucine zipper structure (Lalli and Sassone-Corsi, 1994). The basic domain functions in DNA binding, while the leucine zipper mediates dimerization. This structural motif is also present in the AP-1 constituents c-*fos*, c-*jun*, and their related family members. PDGF or serum stimulation of VSMC induces the dimerization potential of these proteins via phosphorylation by activated protein kinase C. Thus, there are many possible dimers that can be formed in response to activation of PKA and PKC (Hai and Curran,1991). Adding to the complexity of the interaction between these two pathways, the AP-1 complex has been shown to be capable of activating CRE promoter elements (Sassone-Corsi et al,1990). In addition, Arias et al. (1994) have recently demonstrated that CREB and c-*jun* both interact with and depend on a common factor, CBP (phospho-CREB binding protein), to activate transcription.

In PC12 cells, the CRE also mediates transcriptional activation induced by increased intracellular calcium following membrane depolarization (Sheng et al., 1991; Guadiola-Diaz et al., 1994). It will be important to determine whether expression of SM-20 in neuronal cells is responsive to cellular excitation. It is unknown whether

membrane depolarization and calcium increases in non-neuronal cells, such as muscle cells, regulate gene expression via a CRE (Karin, 1992). Examination of SM-20 regulation in these cell types may thus be useful in addressing this question.

Hypotheses regarding SM-20 regulatory levels

The above data raise several issues regarding the expression of SM-20. These include the elevation of SM-20 in neointimal VSMC, the induction *in vitro* by agonists with differing effects on VSMC growth, and the high levels of expression in post-mitotic cells. Analysis of these situations is instructive in generating hypotheses regarding the significance of SM-20 regulation.

Elevation of SM-20 in neointimal VSMC

Following mitogenic stimulation of cultured VSMC, SM-20 mRNA and protein levels are rapidly increased, and then return to or below baseline by 3 hrs. *In vivo*, SM-20 is markedly elevated in the neointimal VSMC 2 weeks after rat arterial injury, and in the VSMC of human coronary atherosclerotic plaque.

The time course of immediate early gene induction in the vessel wall following balloon injury has generally been found to parallel the regulation *in vitro* by growth factors. This has been demonstrated for *JE*, *KC*, tissue factor (Taubman, 1993a), c-*fos*, *fos*B, c-*jun*, *jun*B, and *jun*D (Miano et al, 1993b), which are rapidly induced after injury, and then return to baseline levels within several hrs. In contrast, PDGF A chain, PDGF β receptor, and TGF-β demonstrate

delayed induction and prolonged elevation following growth factor stimulation of cultured VSMC. The mRNA and protein of these genes are upregulated in neointimal VSMC 2 weeks after arterial injury (Majesky et al, 1990; Majesky et al., 1991) as well as in human plaques (Wilcox et al, 1988).

In vivo, SM-20 demonstrates a pattern of regulation analogous to the immediate-early genes β-actin, thrombospondin, and c-*myc*, whose mRNA levels are increased in neointimal VSMC even 1-2 weeks after arterial injury (Campbell et al 1988; Majack et al., 1987; Miano et al, 1993a). Increased β-actin (Dartsch et al, 1989) and c-myc (Marin et al, 1993) protein have also been demonstrated in human atherosclerotic plaque. Nearly 90% of the smooth muscle cell nuclei in carotid artery plaque samples contained high levels of immunoreactive c-myc protein, in comparison to approximately 15% in the media (Marin et al, 1993). This elevation of c-myc in a large percentage of the plaque smooth muscle cells is consistent with previous findings *in vitro*: proliferating cells contain constant amounts of c-*myc* mRNA (Thompson et al, 1985) and protein (Hann et al, 1985) throughout the cell cycle. Thus, SM-20 may be rapidly regulated following mitogenic stimulation of quiescent VSMC and then chronically elevated in proliferating cells.

Specificity of SM-20 response

An interesting aspect of SM-20 regulation in cultured VSMC is that the mRNA levels are induced by hypertrophic, hyperplastic, and growth inhibitory agonists. The immediate early genes are often induced during seemingly disparate cellular responses such as

proliferation and differentiation of PC12 cells (Greenberg et al, 1985). Several hypotheses have been advanced regarding the mechanism by which induction of a common set of genes can determine the different patterns of gene expression and modulate cellular function (Herschman, 1991). The amplitude and time course of induction of a particular gene may vary slightly between different agonists. These differences would then be magnified if a cellular response depended on the combinatorial action of several immediate early genes. This type of regulation is exemplified by the fos and jun transcription factor families in which multiple members are induced by growth stimulation of quiescent cells (Kovary and Bravo, 1991). The c-*jun*/*jun*B heterodimer functions as a transcriptional repressor, whereas the c-jun/ c-fos heterodimer activates transcription (Schutte et al, 1989). Alternatively, post-translational modifications of these proteins may vary. This could lead to differences in the subcellular localization or activity of the protein. In PC12 cells, c-*fos* is induced by both NGF stimulation and membrane depolarization, but is much more highly phosphorylated in response to NGF (Curran and Morgan, 1986). This may have functional consequences for the c-*fos* protein in terms of its DNA binding, dimerization, and transactivation properties. The functional consequences of the SM-20 response to different agonists may be determined by similar mechanisms.

Expression of SM-20 in non-proliferative tissue: Striated muscle

Unlike other immediate-early genes, SM-20 is expressed at high levels in adult rat striated muscle. Myogenesis requires withdrawal from the proliferative cell cycle into the G0-G1 phase (Nadal-Ginard, 1978). Terminally differentiated striated muscle usually express low levels of growth related genes (Jackson et al., 1990; Li and Olson, 1992). In fact, ectopic expression in transgenic mice of the c-*myb* proto-oncogene in skeletal and cardiac muscle actually leads to their degeneration (Furuta et al, 1993). c-*myc*, c-*fos*, c-*jun*, *fos*B, and *Jun*B antagonize muscle-specific gene expression (reviewed by LI and Olson, 1992), whereas anti-sense c-*fos* transcripts allow trans-activation of the MCK promoter by MyoD in normally non-permissive HeLa cells (Weintraub et al, 1989). Myogenic differentiation can be blocked by the expression of c-*jun* and c-*fos* (Pedraza et al, 1994; Bengal et al, 1992). Furthermore, MyoD directly represses transcription of c-*fos* (Trouche et al, 1993). Interestingly, SM-20 is elevated in neointimal VSMC relative to the medial cells. The former have undergone phenotypic modulation, and possess fewer of the properties of differentiated, contractile smooth muscle.

Two hypotheses are proposed to reconcile the induction of SM-20 in proliferative VSMC *in vitro* and *in vivo* with the expression in differentiated striated muscle. The first is based on the assumption that there is only one isoform of SM-20, whereas the second predicts multiple isoforms.

Hypothesis 1: Progressive increases in SM-20 levels accompany differentiation and subsequent growth.

Northern blot and Western blot analysis have revealed one major SM-20 mRNA and protein species expressed in different cell types under a variety of conditions. This supports, but certainly does not prove, the notion that there is only one SM-20 isoform. Therefore, the following model is proposed (see figure 24):

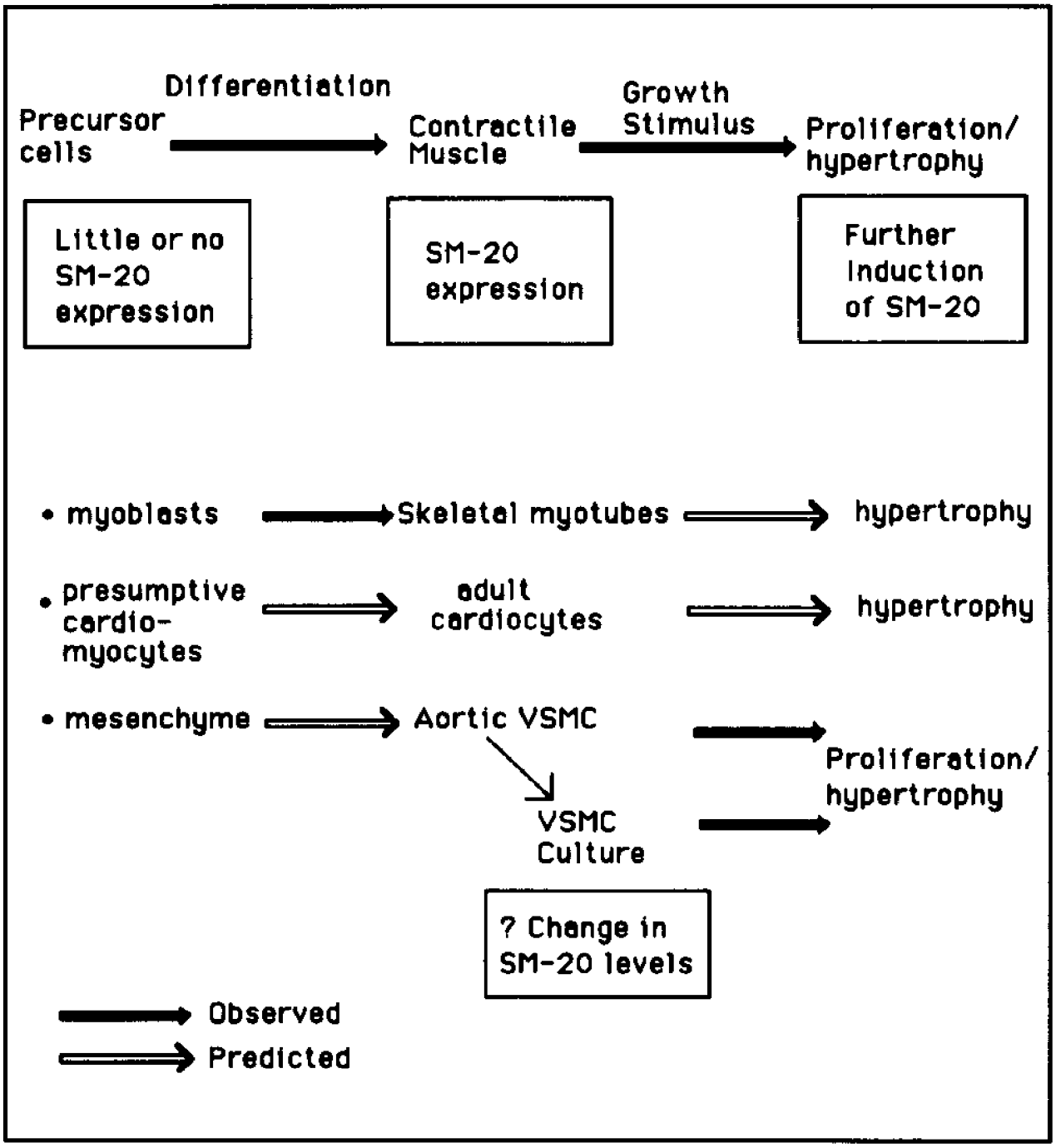


Figure 24. A model for progressive increases in SM-20 levels during differentiation and subsequent growth.

Differentiation of muscle cells may be associated with a basal level of SM-20 expression. The growth of these cells may then induce higher levels of expression. The adult VSMC used in this project, although phenotypically modulated, have undergone differentiation during development of the rat. Quiescent VSMC *in vitro* and *in vivo* thus express baseline levels of SM-20. These levels are then increased by growth factors or injury. Skeletal muscle cells terminally differentiate and express SM-20. It is therefore important to determine whether SM-20 is increased during skeletal muscle hypertrophy.

Although immediate-early genes are usually repressed in differentiated striated muscle, they are often induced by hypertrophic growth stimulation. In cardiocytes, the baseline mRNA levels of the immediate-early genes are very low, but are rapidly increased *in vivo* and *in vitro* after the onset of mechanically- or humorally-induced hypertrophic growth (Chien et al, 1991). These genes include c-*fos*, c-*jun*, *egr*-1, c-*myc*, and *jun*B (Komuro et al., 1990; Izumo et al,1988; Starksen et al., 1986; Iwaki et al, 1990; Chien et al, 1991). Activation of the proto-oncogene H*Ras* is necessary for the cardiocyte hypertrophic growth response to alpha agonist stimulation (Thorburn et al, 1993). The above model predicts that striated muscle hypertrophy may be associated with the rapid induction of SM-20 levels.

Egr-1 and c-*ski* exemplify genes which function in both proliferation and differentiation. *Egr*-1 is an immediate-early gene activated by mitogens in fibroblasts and smooth muscle, yet its upregulation in HL-60 cells drives differentiation into macrophages

(Nguyen et al, 1993). c-*ski* can positively affect a muscle specific function as well as proliferation. The v-*ski* oncogene was first identified by Stavnezer et al (1981) as the product of an avian retrovirus that caused transformation of quail embryo fibroblasts. However, it was later found that upon withdrawal of growth factors, 70-90% of these cells differentiated into fused myotubes, compared to only a few percent of the uninfected cells (Colmenares and Stavnezer, 1989). Thus the induction of the v-*ski* oncogene committed these transformed cells to the myogenic lineage. Overexpression of the cellular proto-oncogene, c-*ski*, also caused this same effect (Colmenares et al, 1991). The normal cellular function of ski is unclear. c-ski is a nuclear protein that binds DNA, but shows no homology to the MyoD family and is not specific to skeletal muscle (Sleeman and Laskey, 1993). SM-20 may have similar properties, affecting both VSMC proliferation and skeletal muscle hypertrophy.

Examination of SM-20 expression in cardiomyocytes *in vivo* and *in vitro* can be used to test the above model. *In situ* hybridization and immunohistochemistry of rats at successive stages of development can be used to establish whether SM-20 is induced during cardiocyte differentiation. These studies can also be used to confirm that SM-20 expression is induced during skeletal myocyte differentiation. The adult heart has already been shown to contain SM-20 mRNA; immunostaining is currently being used to determine the distribution of SM-20 protein in these hearts. The time course of expression of SM-20 mRNA and protein during cardiocyte hypertrophy can be determined by Northern and Western blot analyses. *In vitro*, cultured neonatal cardiocytes can be stimulated

with α-adrenergic agonists, such as phenylephrine. *In vivo*, right and left ventricular hypertrophy can be induced by ligation of the pulmonary arteries and aorta, respectively.

Hypothesis 2: Multiple isoforms account for the induction of SM-20 under various circumstances.

Many of the contractile proteins possess multiple isoforms encoded by either multigene families, such as myosin heavy chain (Mahdavi et al, 1987) and actin (Nudel et al, 1984), or by alternative splicing of a single gene, such as α-tropomyosin (Wieczorek et al, 1988). Expression of these isoforms is regulated according to tissue type and developmental stage.

During the growth of adult muscle, isoforms expressed in fetal stages are often re-induced. Hypertrophic growth of adult cardiocytes is associated with the activation of the skeletal α-actin (Schwartz et al., 1986b) and β-myosin heavy chain genes (Mahdavi et al, 1987; Izumo et al, 1987). Phenotypic modulation of smooth muscle in the neointimal plaque and in culture is associated with the expression of β-actin (Campbell et al, 1988) as well as embryonic myosin heavy chain (Kuro-o et al, 1991).

The regulation of isoform expression is likely to have functional consequences. The non-muscle myosin heavy chain isoform has been associated with cytokinesis in *Dictyostelium* (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987) and in VSMC (Simons and Rosenberg, 1992b), whereas the muscle isoforms are involved in contraction. It is therefore possible that contractile smooth muscle and terminally differentiated striated muscle express muscle-specific

SM-20 isoforms, whereas the neointimal VSMC express a nonmuscle (embryonic) isoform. A determination of the SM-20 genomic structure and the production of exon-specific probes for nuclease protection assays should help resolve this issue. In addition, various isoforms might have different isoelectric points. It might therefore be possible to resolve and detect SM-20 isoforms by immunoblotting of two-dimensional protein gels.

Expression of SM-20 in non-proliferative tissue:

Brain

SM-20 mRNA was detected in the adult rat brain, which is composed largely of post-mitotic tissue. Similarly, c-fos protein is detected in the brain under normal conditions, and is rapidly induced to high levels by neuronal excitation (Sagar et al, 1988). *c-myc* and β-actin are transcribed in differentiated PC12 cells and are rapidly induced by neurotransmitter or NGF stimulation (Greenberg et al, 1985; Greenberg et al, 1986). SM-20 may demonstrate a similar pattern of regulation: baseline levels of expression under normal conditions and induction by neuronal stimulation.

A possible cellular function for SM-20

SM-20 demonstrates a preponderance of expression in smooth, skeletal, and cardiac muscle as well as the brain. SM-20 is not detected in fibroblasts and is markedly induced following myogenic differentiation. These data raise the possibility that SM-20 is associated with cellular excitation. In VSMC, the expression of SM-20

is growth related. In addition, SM-20 protein may be localized to the outer nuclear membrane/endoplasmic reticulum. A cellular process that is involved in both growth and excitation is the mobilization of intracellular calcium from modified portions of the endoplasmic reticulum (Berridge 1993). SM-20 may therefore be involved in calcium release, re-uptake or sequestering. Regions of homology to the EF-hand calcium binding domain consensus sequence were not found within the SM-20 peptide. However, SM-20 may serve an accessory function to other calcium binding proteins.

Proteins involved in the control of intracellular calcium levels have previously been found to be regulated during the growth and differentiation of muscle. The expression of the calcium release channel of the sarcoplasmic reticulum is increased under conditions that induce the partial differentiation and expression of skeletal muscle specific contractile proteins in the myogenic cell line BC3H1 (Marks et al, 1991). Waldron et al (1994) recently found that functional endoplasmic reticulum calcium pumps were necessary for the proliferation of smooth muscle cells, and that the addition of fresh serum appeared to induce the synthesis of this calcium pump. Kent and coworkers (1993) reported the induction of the sarcolemmal Na^{+}-Ca^{2+} exchanger mRNA within one hr of right ventricular pressure overload (a hypertrophic stimulus).

Chapter 8: Significance

SM-20 expression is regulated by growth factors in VSMC culture and is markedly upregulated in the neointimal VSMC. It is therefore possible that SM-20 is critical for VSMC growth or migration in the vessel wall. Neointimal thickening is promoted by VSMC growth and inhibited by the growth and reformation of an endothelial lining. The endothelial lining provides a non-thrombotic surface and secretes inhibitors of VSMC growth. Because the expression of SM-20 appears to be restricted to the VSMC of the vessel wall, SM-20 represents a potentially useful means of targeting therapies to the VSMC while sparing the endothelium. Therapies may be designed to specifically inhibit the synthesis or functioning of SM-20 protein. Alternatively, gene therapy may be employed in which SM-20 regulatory elements control the expression of growth inhibitors or antisense transcripts of growth promoters.

It is likely that the currently used immunocytochemical reagents, such as the monoclonal antibody HHF35 which recognizes muscle-specific actin, do not detect all of the smooth muscle-derived cells of the neointima (Gordon et. al., 1990; Wilcox et al, 1988). The expression of SM-20 may serve as a useful marker to identify VSMC throughout the vessel wall.

SM-20 demonstrates low homology to previously reported sequences, yet its amino acid sequence appears to be highly conserved. The study of SM-20 function may thus reveal critical cellular processes which have not been previously characterized. These processes may be of significance because they are particular to the VSMC growth response and not fibroblasts. In addition, the

striking induction of SM-20 in differentiated skeletal muscle warrants further study of its cellular function in these cells.

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