THE ROLE OF GAS6-AXL IN VASCULAR BIOLOGY

by

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February 2006

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Your file Votre référence
ISBN: 978-0-494-27786-7
Our file Notre référence
ISBN: 978-0-494-27786-7

For my parents, Enver and Fatima.

ABSTRACT

Gas6, the product of a growth arrest specific gene, is a member of the vitamin K-dependent family of γ-carboxylated proteins and the ligand for the tyrosine kinase receptor Axl. Gas6-Axl interactions are important in many biological functions such as cell survival, mitogensis and regulation of thrombosis. We show that gas6 plays animportant role in endothelial cell survival and that its post-translational modification (γ-carboxylation) is necessary for its biologic activity. Using flow cytometry, we first demonstrate that gas6 can prevent apoptosis induced by serum starvation of primary cultures of human umbilical vein endothelial cells (HUVECs). This effect is mediated through activation of the phosphatidylinositol 3-kinase (PI3 kinase) and Akt pathway, known anti-apoptotic regulators. In addition, in the presence of the PI3 kinase inhibitor, wortmannin, gas6 is unable to induce Akt phosphorylation during serum-stress resulting in endothelial cell apoptosis. Similarly, dominant negative Akt constructs prevent gas6-induced endothelial cell survival, underscoring the importance of Akt activation in gas6-mediated survival.

Several downstream regulators of this survival pathway were also identified in HUVECs, namely, NF-κB as well as the antiapoptotic and proapoptotic proteins Bcl-2 and caspase 3, respectively. Luciferase assay experiments indicate that gas6 induces a rapid increase in NF-κB transcriptional activity. We also show that NF-κB is phosphorylated early after gas6 treatment as evidenced by Western Blot analysis. As well, the level of Bcl-2 protein increased, supporting the notion that the Bcl-2

antiapoptotic pathway is stimulated, and levels of caspase 3 activation, a know pro-apoptotic regulator, are significantly reduced with gas6 treatment. These initial results indicate that gas6-Axl interactions are an important mediator of endothelial cell survival during serum stress through activation of classical antiapoptotic pathway, namely, Akt phosphorylation, NF-κB activation, increased Bcl-2 protein expression, and a reduction in caspase 3 activation.

A most intriguing feature of gas6 as a mediator of cell survival is its unusual posttranslational modification, γ-carboxylation. Thus, understanding the role of Gla domain of gas6 in gas6-Axl interaction is of fundamental importance since γ-carboxylation may influence the anti-apoptotic property of gas6-Axl interaction. Here for the first time we show that Gla domain of gas6 is indispensable for its anti-apoptotic function. Initially, we show that carboxylated gas6 binds to phosphatidylserine-containing phospholipid membranes in an analogous manner to other γ-carboxylated proteins whereas decarboxylated gas6 does not. Further, the γ-carboxylation inhibitor, warfarin, abrogates gas6-mediated protection of NIH3T3 fibroblasts from serum starvation-induced apoptosis. A similar effect is observed on endothelium where only carboxylated but not decarboxylated gas6 protects endothelial cells from serum starvation-induced apoptosis. In addition, we also demonstrate that proper γ-carboxylation of gas6 affects activation of its downstream targets, Axl and Akt . These results thus clearly indicate that this post-translational modification imparts a significant role in function of gas6.

Therefore, we propose that gas6 plays important role in endothelial cell survival and that its post-translational modification, γ-carboxylation, is crucial for this function.

RÉSUMÉ

Gas6 est le produit d'un gène spécifique d'arrêt de croissance (GAS), et un membre de la famille de protéines γ-carboxylées qui sont dépendantes de la vitamine K. Gas6 est le ligand pour le récepteur tyrosine kinase Axl. L'intéraction gas6-Axl est importante dans plusieurs fonctions biologiques telle que la survie cellulaire, la mitogénèse et la régulation de la thrombose. Nous démontrons ici que gas6 joue un rôle important dans la survie des cellules endothéliales et que sa modification post-translationnelle, la γ-carboxylation, est nécessaire pour son activité biologique. À l'aide de la cytométrie de flux, nous montrons premièrement que gas6 peut prévenir l'apoptose induite par la carence en sérum des cultures primaires de cellules endothéliales humaines de la veine de cordon ombélical (HUVECs). Cet effet de survie est modulé à travers l'activation de la kinase phosphotidylinositol 3 (PI3 kinase) de même que l'activation de la voie de signalisation de AKT, deux régulateurs connus de l'anti-apoptose. De plus, en présence de la wortmannine, un inhibiteur de la PI3 kinase, gas6 est incapable d'induire la phosphorylation de AKT lors de la carence en sérum ce qui résulte en l'apoptose des cellules endothéliales. De façon similaire, le AKT dominant négatif empêche la survie induite par gas6 chez les cellules endothéliales, soulignant l'importance de l'activation de AKT dans la survie induite par gas6.

Plusieurs régulateurs en aval de cette voie de survie ont aussi été identifiés dans les HUVECs, soit NF-κB, de même que la protéine anti-apoptotique Bcl-2 et la protéine pro-apoptotique caspase 3. L'activité luciférase indique que gas6 induit une augmentation rapide de l'activité transcriptionnelle de NF-κB. Nous démontrons aussi que NF-κB est phosphorylé tôt après le traitement avec gas6 tel qu'illustré par l'analyse

de type western blot. De plus, le niveau de la protéine Bcl-2 est augmenté, supportant la notion que la voie anti-apoptotique de Bcl-2 est stimulée et le niveau d'activation de caspase 3, un régulateur pro-apoptotique est réduit de façon significative lors du traitement avec gas6. Ces résultats initiaux indiquent que l'intéraction gas6-Axl joue un rôle de médiateur important dans la survie des cellules endothéliales soumises à la en sérum. Ce rôle est effectué à travers l'activation de voies anti-apoptotiques classiques telles que la phosphorylation de AKT, l'activation NF-κB, l'augmentation de l'expression de la protéine Bcl-2 et la réduction de l'activation de caspase3.

Une des charactéristiques les plus intriguante de gas6 en tant que médiateur de survie cellulaire est sa γ-carboxylation, une modification post-translationnelle peu commune. Ainsi, une meilleure compréhension du rôle du domaine Gla de gas6 dans l'intéraction gas6-Axl est d'une importance fondamentale puisque la γ-carboxylation pourrait influencer la fonction anti-apoptotique de l'intéraction gas6-Axl. Ici, pour la première fois, nous démontrons que le domaine Gla de gas6 est indispensable pour sa fonction anti-apoptotique. Initiallement, nous montrons que le gas6 carboxylé se lie à des membranes de phospholipides composées de phosphatidylserine de façon analogue aux autres protéines γ-carboxylées alors que le gas6 décarboxylé ne peut se lier à ces mêmes membranes. De plus, l'inhibiteur de γ-carboxylation warfarine bloque l'effet de protection contre l'apoptose de gas6 chez les fibroblastes NIH3T3 soumis à la carence en sérum. Un effet similaire est observé sur l'endothélium où seulement le gas6 carboxylé et non pas le décarboxylé protège les cellules endothéliales contre l'apoptose induite par la carence en sérum. De plus, nous démontrons que la γ-carboxylation adéquate de gas6 affecte l'activation en aval de ses deux cibles, soit Axl et AKT. Ces résultats indiquent

clairement que cette modification post-translationnelle joue un rôle significatif dans la fonction de gas6.

Nous proposons donc que gas6 possède un rôle important dans la survie des cellules endothéliales et que sa modification post-translationnelle, la γ-carboxylation, est cruciale pour cette fonction.

PREFACE

This Ph.D. thesis was written in accordance with the Guidelines for Thesis Preparation from the Faculty of Graduate Studies and Research at McGill University. I have exercised the option of writing the thesis in manuscript style. According to the guidelines, ".....candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the 'Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. ....the **thesis must be more than a collection of manuscripts**. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory. .....the thesis must include the following: (a) a table of contents; (b) an abstract in English and French; (c) an introduction which clearly states the rational and objectives of the research; (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (e) a final conclusion and summary.....in addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled 'Contributions of Authors' as a preface to the thesis. ....."

I have included, as chapters of this thesis, the texts of two original papers, both of which have been published. Each of the aforementioned chapters contains its own introduction, materials and methods, results, discussion and references section. A general

introduction and literature review is given in Chapter 1 and a final discussion is presented in chapter 4. The references for Chapters 1 and 4 are included at the end of the thesis.

Papers included in this thesis:

Chapter 2 **Hasanbasic, I.,** Cuerquis, J., Varnum B., and Blostein, MD. Intracellular signaling pathways involved in Gas6-Axl-mediated survival of endothelial cells. AJP – Heart, 287:1207-1213, 2004.

Chapter 3 **Hasanbasic, I.**, Rajotte, I., and Blostein, M. The role of γ-carboxylation in the anti-apoptotic function of gas6. J Thromb Haemost., 3: 2790–7, 2005.

Contributions of Authors:

The candidate performed most of the research included in this thesis. Contributions of other authors to this work are described as follows: Cuerquis, J., performed wortmannin experiments described in Chapter 2; Rajotte, I., performed gas6 purification and assisted in fluorescence and flow cyrometry experiments described in Chapter 3. Rajotte, I., assisted in flow cytometry experiment as shown in Chapter 4.

ACKNOWLEDGMENTS

I would like to express my gratitude to my supervisor, Dr. Mark D. Blostein, for his encouragement, patience and support during the course of my Ph.D. study. His commitment to the advancement of medical science has been motivating factor in my studies.

I would also like to thank my fellow graduate students, postdoctoral fellows and technicians who have contributed with lively scientific discussions and a supportive environment. In particular, I would like to thank Dr. Cho, Jessica Cuerquis and Isabelle Rajotte for their immense assistance.

I would like to thank Isabelle Rajotte for assisting in translation of the Abstract into the French language.

I would also like to acknowledge the Canadian Hearth and Stroke Foundation for PhD studentship support (2001-2006).

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AGC	cAMP-dependent protein kinase A/ protein kinase G/ protein kinase C family
AIF	Apoptosis-inducing factor
AKT(PKB)	Kinase, protein kinase B
AoSMC	Aortic smooth muscle cells
APC	Antigen-presenting cell
ATP	Adenosine Triphosphate
AxlECD	Axl, extracellular domain
Axl-ECD/TM	Axl extracellular domain and transmembrane domain
bFGF	basic fibroblast growth factor
BH	Bcl-2 homolgy domain
cAMP	Adenosine 3':5' cyclic-monophosphate
CNS	Central nervous system
CREB	cAMP response element binding proteins
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetate
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated death domain
FN III	Fibronectin type III
Gas6	Growth arrest specific 6
Gla	Gamma-carboxyglutamic acid
GN	Glomerulonephritis
GnRH	Gonadotropin-releasing hormone
Gsk-3β	Glycogen synthase kinase-3β
HUVEC	Human umbilical vein endothelial cell
IgG1	Immunoglobulin G1

IL-1β	Interleukin-1β
IL-8	Interleukin-8
JNK	c-Jun-N-terminal- Kinase
K_D	Dissociation constant
LDL	Low density lipoprotein
LG	Laminin globular
M07e	A human leukemic factor-dependent cell line
MAP	Mitogen-activated kinase
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor kB
OS	Outer segment
OxLDL	Oxidized low density lipoprotein
PAF	Platelet-activating factor
PDGF-B	Platelet-derived growth factor-B
PDK1	Phosphoinositide-dependent kinase 1
PH	Pleckstrin homology
PI3K	Phosphotidyl inositol 3-kinase
PIP3	Phosphatidylinositol (3, 4, 5) trisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PS	Phosphatidylserine
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
RPE	Retinal pigment epithelium
RTK	Receptor tyrosine kinase
SRA	Scavenger receptor A
STAT	Signal transducers and activators of transcription
STZ	Streptozotocin
TGF-β	Sransforming growth factor-β
TNF	Tumor necrosis factor

v-Akt	Viral protein kinase B
VEGF	Vascular endothelial growth factor
VKOR	Vitamin K_1 2,3-epoxide reductase
VSMC	Vascular smooth muscle cells

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Cell clearance is necessary for normal development (Meier et al., 2000), and maintenance of tissue homeostasis in a mammalian organism (Hengartner, 2000). Such cell clearance can be executed by two fundamentally different processes, necrosis and apoptosis (Wyllie et al., 1980). Necrosis, a passive destruction of a cell, is mainly initiated by extrinsic factors such as osmotic, toxic, thermal, traumatic, and hypoxic-ischemic insults (Majno and Joris, 1995; Buja et al., 1993). It is characterised by a loss of cytoplasmic membrane integrity and a rapid influx of Na^{+}, Ca^{2+}, and water, leading to cytoplasmic swelling and nuclear pyknosis (Barros et al., 2001; Berridge et al., 2000). These events are followed by cellular fragmentation and release of lysosomal and granular contents into the surrounding extracellular space resulting in inflammation (Padanilam et al., 2003; Scaffidi et al., 2002).

Conversely, apoptosis or a programmed cell death, an active energy-requiring program, is defined by cytoplasmic and nuclear shrinkage, chromatin condensation, fragmentation, and formation of apoptotic bodies (Kerr et al., 1972). Apoptosis is essential for multicellular animals since it allows the organism to tightly control cell numbers and protect itself from potentially dangerous cells that threaten homeostasis (Hengartner, 2000). This type of programmed cell death is observed in various cell types

and is mediated by the absence of survival factors or presence of death-promoting factors (Stoneman and Bennett, 2004).

Recently, a novel survival factor, gas6, and its receptor Axl have been implicated in the protection of serum starved NIH 3T3 fibroblasts from cell death by apoptosis induced by complete growth factor depletion and mediated through activation of PI3 kinase (Goruppi et al., 1997). Gas6-Axl protein-protein interactions play a role in nephrotoxic glomerular injury (Yanagita et al., 1999), in mediation of thrombosis (Angelillo-Scherrer et al., 2005) in immune responses (Lemke and Lu, 2003; Lu and Lemke, 2001) and in vascular remodeling (Melargano et al., 1999), all of which will be addressed in this review. More importantly, the unique posttranslational modification of gas6 on its N-terminus, called γ-carboxylation, will be examined in detail since gas6 represents the first γ-carboxyglutamic acid (Gla) domain containing ligand that has been shown to regulate cell activity.

Finally, this review will elaborate on literature concerning apoptosis as it relates to the gas6-Axl protein-protein interactions as well as the current state of knowledge involving these particular interactions in various cell systems and disease states.

1.2 Apoptosis or programmed cell death

Apoptosis is a regulated process, initiated by the absence of growth factors and/or presence of death-promoting factors. All cells contain components of the death machinery, ready to initiate self-execution unless signaled not to do so. In fact, death is often the default program unless the cell is actively signaled to survive (Stoneman and Bennett, 2004). Once a death signal is initiated, a cell goes through a series of

morphological changes, beginning from shrinkage of the cell membrane, condensation of nuclear chromatin, cellular fragmentation and, finally, formation of apoptotic bodies. The apoptotic bodies may be phagocytosed by adjacent cells, and are consequently removed without significant inflammation or formation of scar tissue (Kerr et al., 1972).

Cell apoptosis can be regulated through two biochemically distinct pathways, a mitochondria-independent pathway and a mitochondria-dependant pathway (Hengartner, 2000).

1.2.1 Mitochondria-independent pathway

The mitochondria-independent pathway, critical for immune selection and inflammation (Krammer, 2000), is initiated by ligation of cell surface death receptors, such as the CD95 (Fas) and the TNF-family of receptors (death receptors) (Ashkenazi and Dixit, 1998). The death ligand–receptor interaction results in recruitment of a number of adapter proteins, including FADD (Fas-associated death domain), through protein-protein interactions, and subsequently promotes the activation of upstream caspase 8 (Cardier and Erickson-Miller, 2002; Pru et al., 2003).

1.2.2 Mitochondria –dependent pathway

The mitochondria-dependant pathway is cytochrome c dependant and is characterized by increased mitochondrial leakiness. This results in a collapse of mitochondrial function leading to cytoplasmic release of factors normally found in the space between the inner and outer mitochondrial membranes (including cytochrome c, AIF, and others) which subsequently facilitates caspase activation (Wang, 2001). The

apoptotic signal is then relayed downstream via the assembly of a complex, called the apoptosome, which promotes the activation of upstream caspase-9 by limited proteolysis or allosteric rearrangement (Kroemer and Reed, 2000).

In order to induce apoptosis, caspases first need to be activated through enzyme-dependant cleavage of inert zymogens. Cleavage of inactive zymogens leads to formation of active enzyme (Thornberry et al., 1997). Once activated, caspase 9 activates three short prodomain caspases, caspase 3, 6 and 7 which are considered final executioners of the caspase cascade and are usually more abundant and active than their prodomain counterparts (Buendia et al., 1999).

Based on their function in apoptosis, the caspases have been divided into two groups: initiators and effectors. Initiator caspases (caspases 2, 8, 9, and 10) activate effector caspases (caspases 3, 6, and 7) (Johnson, 2000). These effector caspases in turn directly degrade multiple substrates including the structural and regulatory proteins in the cell nucleus, cytoplasm, and cytoskeleton leading to cell death (French and Tschopp, 2003).

Caspase-induced apoptosis can be triggered by various apoptotic stimuli which can directly or indirectly influence the permeability of the outer mitochondrial membrane ultimately leading to the release of cytochrome c. The cytoplasmic efflux of cytochrome c is the key event in activation of the mitochondria-dependant pathway (Cory et al., 2003). Cytochrome c release is initiated by the bcl-2 family of proteins and depends on the ratio between prosurvival (Bcl-2, Bcl-xL, Bcl-w etc.) and proapoptotic proteins (Bax, Bak, Noxa etc.) (Guo et al., 2002).

1.2.3 Bcl-2 family of proteins

The Bcl-2 family of proteins is an important regulator of caspase activation. The life-or-death decisions of these proteins are regulated by the balance between its anti- and pro-apoptotic members (Cory and Adams, 2002). There are 20 members of the Bcl-2 family in mammals, all of which share at least one conserved Bcl-2 homology (BH) domain (Oltvai et al., 1993). Proapoptotic Bcl-2 family of proteins, namely, Bax, Bak, and Noxa, play a role in mitochondria disruption and act as damage sensors, directly antagonizing prosurvival proteins (Heibein. et al., 2000).

Conversely, the prosurvival Bcl-2 family of proteins, Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1, all inhibit apoptosis during development and in response to cellular stress by inactivating their antiapoptotic counterparts (Borner, 2003). They are composed of three conserved BH (Bcl-2 homology) domains. Three of these (BH1, BH2 and BH3) fold together to form a hydrophobic groove on the prosurvival molecules (Muchmore et al., 1996). This groove is the target for binding to proapoptotic Bik/Nbk/Blk, Bid, Bad, ^{Bim}/Bod, Noxa, and Bcl-G_s also known as BH3-only proteins.

There are two groups of Bcl-2 family of proteins that promote cell death: Bax and BH3-only families (Adams and Cory, 1998). Members of Bax family have sequences that are similar to those in Bcl-2, especially in BH1, BH2 and BH3 regions, whereas BH3 only proteins contain only a BH3 motif that serves as an interaction domain necessary for their killing action (Huang and Strasser, 2000). Both, Bax and BH-3 only families are essential for initiation of apoptosis whereas BH-3 only proteins act as damage sensors

and direct antagonists of the prosurvival proteins whereas Bax-like proteins act further downstream in mitochondrial disruption (Gross et al., 1999).

Thus, in the presence of apoptotic signals, BH3-only proteins are released to bind and inactivate prosurvival Bcl-2 proteins (Huang and Strasser, 2000). Once released they abrogate the activity of the prosurvival Bcl-2 family of proteins and, with the help of Bax-like proteins, induce the subsequent release of cytochrome c (Martinou and Green, 2001).

Inactivation of proapoptotic Bcl-2 family of proteins can be regulated by upstream pathways involving PI3 kinase and Akt (Brazil and Hemmings, 2001). Recently, a proapoptotic member of the Bcl-2 family, Bad, was found to be a substrate of Akt (Datta.et al., 1997). Once activated, Akt phosphorylates Bad at Ser 136, which allows Bad to associate with 14-3-3 protein and remain sequestered in the cytoplasm. As a result, the proapoptotic functions of Bad are inactivated. Conversely, when in the dephosphorylated state, Bad can form heterodimers with the Bcl-2 family anti-apoptotic proteins like Bcl-xl and inactivate them allowing subsequent cytochrome c release (del Peso et al., 1997). Thus, one of the important activators of the Bcl-2 family of proteins appears to be Akt.

1.3 AKT/PI3 Kinase Pathway

The activation of the Akt pathway provides cells with a survival signal that allows them to withstand apoptotic stimuli (Yao and Cooper, 1995). Once activated by PI3

kinase, Akt (PKB) has direct effects on the apoptosis pathway by targeting numerous anti- and pro-apoptotic molecules.

1.3.1 PI3 Kinase

Numerous studies have implicated PI3 kinases and their phospholipid products in promoting survival downstream of extracellular signals. Survival stimuli generally mediate signaling through activation of transmembrane receptors, which may possess intrinsic tyrosine kinase activity, or are indirectly coupled to tyrosine kinases or transmembrane G protein coupled receptors (Clark and Brugge, 1995; Segal and Greenberg, 1996; Weiner and Chun, 1999).

Although there are several types of PI3 kinases, class I enzymes are primarily responsible for the production of D-3 phosphoinositides necessary for the initiation of cell survival pathways (Fruman, 1998). Class I PI3 kinase enzymes are composed of regulatory and catalytic heterodimers. The catalytic domain is located at the carboxy-terminus while the regulatory subunit binding domain is located at amino-terminus (Wymann, 1998). The activity of the catalytic subunit (p110) is regulated by the regulatory subunit (p85) and is maintained in a low-activity state in quiescent cells. P110 subunit activation is mediated by direct interaction with phosphotyrosine residues of activated growth factor receptors or adaptor proteins. The catalytic subunit can also directly bind to Ras protein and further stimulate PI3 kinase activity. Its activity can be irreversibly inhibited by wortmannin at low nanomolar concentrations through Schiff base formation with a lysine in the kinase domain (Wymann, 1996).

Following ligand binding, receptor tyrosine kinases are activated and, as such, recruit PI3 kinase isoforms to the inner plasma membrane (Toker and Cantley, 1997;

Rameh and Cantley, 1999). Once on the plasma membrane, PI3 kinase generates 3'-phosphorylated phosphoinositides, through ATP dependant processes. Isoforms of PI3K that are activated in response to receptor-mediated survival signals principally generate phosphatidylinositol 3, 4 bisphosphate (PI3, 4P) and phosphatidylinositol 3, 4, 5 trisphosphate (PI3, 4,5P). Once generated, these lipids then function as signaling intermediates that regulate downstream signal transduction cascades. Signaling proteins with pleckstrin homology domains (PH) accumulate at sites of PI3 kinase activation by directly binding to PI3, 4,5P via unique lipid-binding motifs. PI3, 4,5P at the membrane brings these proteins in close proximity to other serine-threonine kinases such as Akt and PDK1 and facilitates their phosphorylation (Lawlor, 2001). Consequently, phosphorylation of Akt results in activation of other proteins that effect cell growth, cell survival and cell cycle entry.

1.3.2 Akt

In recent years, Akt/PKB has emerged as an important signaling molecule activated in response to growth factors or insulin and is thought to contribute to several cellular functions such as nutrient cell growth, transcriptional regulation, metabolism, and cell survival (Brazil and Hemmings, 2001). However, deregulation of this kinase is frequently associated with human diseases including cancer and diabetes.

Initially, Akt/PKB was identified based on its homology to protein kinase A (PKA) (Coffer and Woodgett, 1991) and C (PKC) (Jones et al., 1991) or as the cellular homolog of the retroviral oncogene viral akt (v-Akt) (Bellacosa et al., 1991). Akt/PKB protein kinase, a serine/threonine kinase, belongs to the cAMP-dependent protein kinase

A/ protein kinase G/ protein kinase C family (AGC) (Hajduch et al., 2001). This family of protein kinases share structural homology within their catalytic domain and have the similar mechanism of activation (Song et al., 2005). There are three Akt/PKB genes in mammals: PKBα/Akt1 (Jones et al., 1991), PKBβ/Akt2 (Cheng et al., 1992) and PKBγ/Akt3 (Brodbeck et al., 1999).

Structurally, Akt /PKB is composed of an amino terminal pleckstrin homology (PH) domain, a central kinase domain and carboxyl-terminal regulatory domain that contains a hydrophobic motif (Song et al., 2005). The PH domain of Akt is composed of 100 amino acids and was originally found in pleckstrin, the major substrate for PKC in platelets (Tyers et al., 1998). Recent reports reveal that the PH domain of Akt shares similarity to those found in other signaling molecules that bind 3-phosphoinositides (Lietzke et al., 2000; Ferguson et al., 2000).

Akt activation is dependent on the interaction between its PH domain and activated phosphatidylinositol (3, 4, 5) trisphosphate (PIP3) produced by phosphatidylinositol 3-kinase (PI3-kinase). PH domains are primarily lipid-binding molecules, although they can also be involved in protein-protein interactions (Bottomley, 1998; Lemmon et al., 1996; Rebecchi et al., 1998). Mutation of the Akt PH domain blocks its activation by growth factors *in vivo* (Franke et al., 1995) suggesting that Akt activation is regulated via the binding of PH domain to PIP3. Activation of Akt and its subsequent phosphorylation at Thr308 is mediated by phosphoinositide-dependent kinase-1 (PDK1), with binding of its PH domain to PIP3 in response to growth factor stimulation (Alessi et al. 1997). Activation of Akt by PDK1 depends on availability of PI3 kinase generated PIP3s (phosphatidylinositides). Importance of PH domain in Akt

activation can be further examined by mutation studies demonstrating that an Akt PH domain dominant mutant (Akt R25C), which does not bind phosphoinositides, fails to be phosphorylated by PDK1 *in vitro*. Conversely, deletion of the PH domain allows this mutant (AktΔPH) to be phosphorylated by PDK1 in the absence of added phosphoinositides (Stokoe et al., 1997; Alessi et al., 1997) indicating that PH domain may play negative regulatory role and D3PPIs generated by PI3 kinase may remove its inhibitory effect on phosphorylation (Chan et al., 1999).

1.3.2.1 Three step activation of Akt

Akt activation is a three-step process. The first step is defined by the constitutive phosphorylation of Akt at Thr450 which may be a marker for proper folding of the protein (Alessi et al., 1997). The second step involves translocation of Akt to the plasma membrane, and the third step involves phosphorylation of Akt at Thr308 and Ser473 (Chan et al., 1999). All three steps are interdependent and occur sequentially.

1.3.2.2 Downstream targets of Akt

Once activated, Akt transduces signals that regulate multiple biological processes which include apoptosis, gene expression, and cellular proliferation. Akt activation has been linked to induction of c-myc and Bcl-2 (Ahmed et al., 1997). Phosphorylation of Akt leads to inactivation of Gsk-3β (Cross et al., 1995) and members of the forkhead family of transcription factors (Cichy et al., 1998).

One of the major roles of Akt is its ability to regulate apoptosis. It has been shown that PI3 kinase /Akt pathway activation contributes to the transduction of growth factors

and subsequent protection from apoptosis in a variety of cell types (Dudek et al., 1997; Khwaja et al., 1998; Eves et al., 1998; Kennedy et al., 1997). Akt has been shown to phosphorylate a number of key apoptotic regulators, such as, the NF-κB transcriptional regulator, Bad (Datta et al., 1997), a proapoptotic protein of bcl-2 family, and the forkhead transcription factors (Franke et al,.1999). Furthermore, Akt promotes the activity of the antiapoptotic bcl-2 family of proteins including Bcl-2 and Bcl-x_L. For example, activation of NF-κB by Akt can inhibit apoptosis through induction of antiapoptotic proteins as well as lead to enhanced NF-κB mediated transcriptional activity in many cell lines (Pan et al., 1999; Sizemore et al., 1999; Madge et al., 2000). Controversy still exists about the mechanisms by which Akt activates NF-κB. Some studies show increased nuclear localization of NF-κB (Pan et al., 1999) while others demonstrate no increase in nuclear localization but rather enhanced transcriptional activity upon phosphorylation of its subunit (Madrid et al., 2000). Akt activation can also inhibit the action of pro-apoptotic members of Bcl-2 family of proteins, namely, Bad , by phosphorylating it and directly inhibiting release of cyrochrome c from mitochondria.

On the other hand, Akt itself is a target of numerous growth factors which play role in cell survival. One of those molecules is a recently described member of vitamin K dependant family of proteins, namely growth arrest specific gene 6 (gas6).

1.4 Gas6 and its discovery

Gas6 was initially isolated from growth arrested NIH 3T3 fibroblasts and its expression was dramatically reduced upon induction of cell cycle rentry in both mouse and human fibroblasts (Manfioletti *et* al., 1993). The cell cycle governs the growth of eukaryotic cells. The control of cell proliferation occurs mainly in the G1 phase and growth arrest in this phase can be accomplished by growth factor depletion. This state of growth arrest is called G0. The G0 phase is associated with growth-arrest specific mRNAs whose expression is negatively regulated by growth factor stimuli (Schneider et al., 1988). A set of genes that are highly expressed during serum starvation in NIH3T3 mouse fibroblasts have been isolated (Schneider et al., 1988). One of these genes encodes for growth arrest specific gene 6 (gas6) product (Manfioletti et al., 1993). It is a ligand for the receptor tyrosine kinase Axl (Varnum et al., 1994).

1.4.2 Gas6 structure

Murine and human cDNAs both reveal that gas6 is a novel member of the vitamin K family of proteins and is homologous to protein S, a negative regulator of the blood clotting cascade (Esmon, 1987 & 1989). Gas6 lacks any anticoagulant activity (Evenas et al., 2000). Protein S and gas6 share 43% homology. Both molecules are composed of a Gla domain, four EGF-like repeats, and a carboxy-terminal tandem of two laminin globular (LG) domains with homology to the steroid hormone-binding globulin (Sasaki et al., 2002). The sequence comparison outlined in Figure 1 shows that the highest homology regions of these two proteins are the Gla domain (Region A) and EGF domain (Region C) regions.

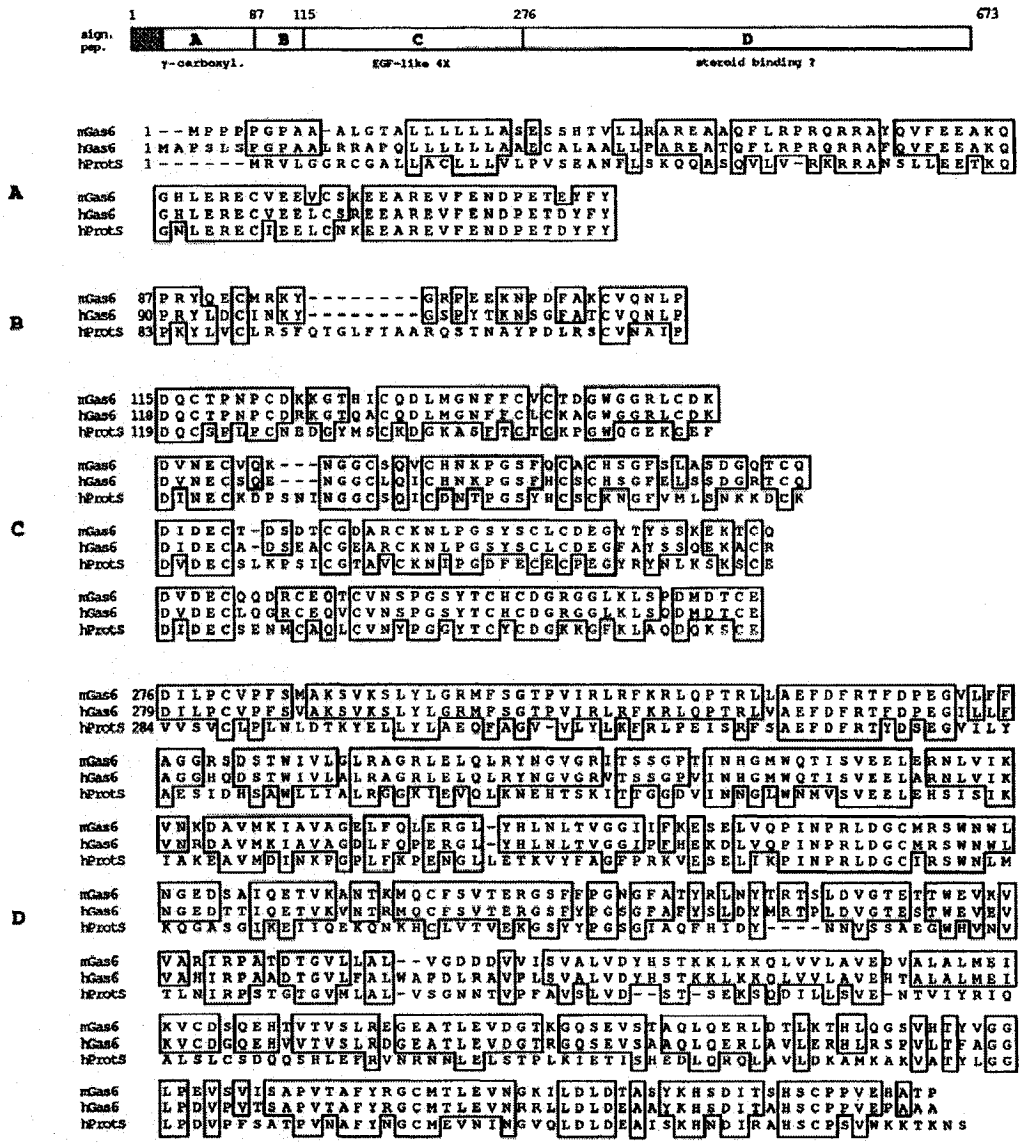


Figure 1-1. Analysis of the gas6 cDNA-encoded protein. The overall organization of the predicted Gas6 sequence and the relative sizes of the four regions in the protein are shown. Amino acid sequences of mGas6, hGas6, and human protein S is compared below. A, B, C, and D refer to the four regions present in these proteins (Sasaki et al., 2002).

1.4.2.1 C-terminus LG domains

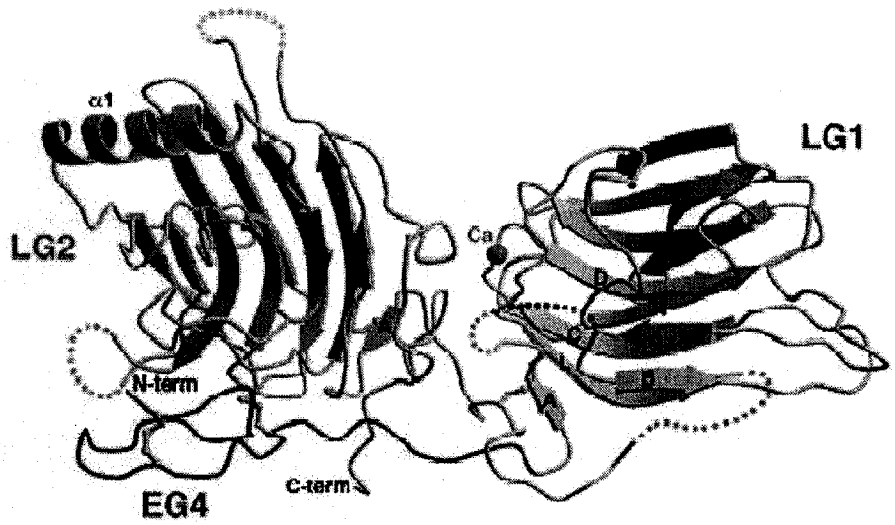
The gas6 C-terminus is composed of two LG domains. LG domains are found in a diverse set of proteins such as extracellular matrix proteins, cell surface receptors, and hormone-binding proteins (Rudenko et al., 2001). LG domains have been found to be important for interactions with proteins, binding glycosaminoglycans and transporting steroid hormones (Hohenester et al., 1999). Experiments with gas6/protein S chimeras demonstrate that the C-terminal LG domains contain a receptor binding site for receptor tyrosine kinases (Nagata et al., 1996). LG domains of gas6 also contain a calcium binding site in the domain interface (Figure 1-1A). This calcium binding site appears to be important in the folding and structural integrity of LG domains.

The C-terminal LG domain of gas6 contains a binding site for the receptor tyrosine kinase Axl family (Sasaki et al., 2002). Gas6 binds to Axl with an affinity (K_D) of 1-4 nM. Binding of gas6 to Axl has been precisely mapped out by Sasaki et al. (2002) and is located in a stretch of solvent-exposed hydrophobic residues located in LG2 domain, in close proximity to the calcium binding site at the junction of the LG1 and LG2 domains. Moreover, mutations of some residues in this stretch, especially Leu 620, significantly reduces the affinity of gas6 for Axl (Figure 1-1B).

In a recent study by Fisher et al. (2005), a novel binding site contributing to gas6 - Axl interaction was mapped out. A monoclonal antibody (CNTO300) was able to recognize a conformationally sensitive epitope and its binding was inhibited by EDTA indicating a requirement for calcium (Figure 1-2). These findings support the hypothesis that the Ca^{2+}-binding site of Gas6 stabilizes the V-shaped conformation held by the LG1

and LG2 domains (Sasaki et al., 2002). Two receptor-binding sites on gas6, a hydrophobic patch on LG2 and a novel site on LG1 are described and supported by the following data: (i) the CNTO300 antibody cannot completely block receptor binding, (ii) the sequence of this epitope is located on the LG1 domain distant from calcium binding site and LG2 hydrophobic patch, (iii) a mixture of CNTO300/Gas6 or CNTO300/LG1 yielded the same antibody sequence indicating that a novel antibody-binding site is located in the LG1 fragment, and (iv) receptors bind to soluble LG1 fragment directly.

A



B

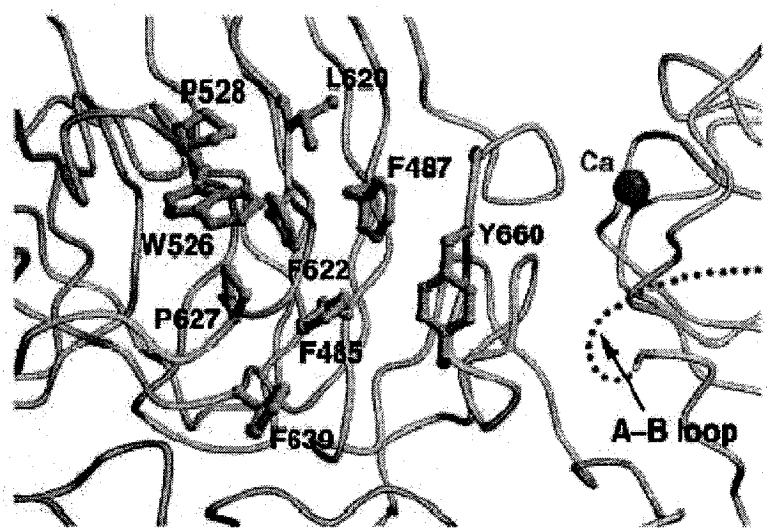


Figure 1-2.Crystal structure of gas6 C-terminus LG domains. A. Structure of LG1 and LG2 domains showing the Ca binding site. B. The solvent exposed hydrophobic region in gas6 C-terminus outlining solvent-accessible hydrophobic residues (Sasaki et al., 2002).

1.4.2.2 The N-terminal Gla domain

The gas6 N-terminus is composed of 12-13 γ-carboxyglutamic residues representing the Gla domain of the molecule (Tanabe et al., 1997). The Gla domain is present in various proteins involved in blood coagulation and plays a crucial role in their function. It is generated by vitamin K-dependent γ-carboxylation of a cluster of Glu residues and is thought to be involved in intermolecular protein–protein or protein–membrane interactions (Furie and Furie, 1988). Such domains are required for calcium-dependent binding to membrane phospholipids, suggesting a possible role of gas6 in cell–cell interactions (Manfioletti et al., 1993).

1.4.3 Role of vitamin K proteins

The Gla domain is necessary for the activity of vitamin K dependent proteins. The known vitamin K-dependent proteins include coagulation factors VII, IX, X, prothrombin, protein Z, protein S and protein C. Gas6 (which functions as a ligand for the Axl family of tyrosine kinase receptors), the bone-related proteins (osteocalcin and matrix Gla protein), and four integral membrane proteins of unknown function are also Gla proteins (Kulman et al., 2001). Even though the vitamin K dependent family of proteins play crucial role in blood coagulation, they also play a significant role in prevention from osteoporosis and arterial calcification (Adams and Pepping, 2005), nephrotoxic glomerular injury (Yanagita et al., 1999), immune responses (Lemke and Lu, et., 2003; Lu and Lemke et al. 2001) and vascular remodelling (Melargano et al., 1999).

Vitamin K dependent coagulation factors function by maintaining the integrity of the mammalian circulatory system after blood vessel injury (Furie and Furie, 1988). The

coagulation system is triggered in response to rupture of the endothelium, which allows exposure of blood to the extravascular tissue (Dahlback, 2000). Once initiated, the blood clotting cascade is dependant on sequential activation of certain plasma zymogens to their active enzyme forms (Davie and Ratnoff, 1964). These plasma proteins, including factor XII, factor XI, factor IX, factor X, factor VII, and prothrombin, are zymogens of serine proteases and are activated when assembled in the complexes on membrane surfaces with protein cofactors.

Initiation of blood coagulation requires the presence of calcium ions that are necessary for the interaction of these proteins with phospholipid membrane. Thus, a platelet plug forms when fibrinogen is converted by thrombin to fibrin that then assembles into a fibrin polymer. Once formed, the clot obstructs the flow of blood from the injured vessel and minimizes blood loss from the wound (MacFarlane, 1964). Hence, blood coagulation and platelet-mediated haemostasis have evolved as important defense mechanisms against bleeding. Under normal conditions, anticoagulant mechanisms ensure control of coagulation and any disturbance of the balance between anticoagulant and procoagulant mechanisms may result in thrombosis or bleeding respectively (Hoffman et al., 1996).

Bleeding and thrombosis may be triggered by platelet plug instability, thus, agents inhibiting the formation of stable platelet plugs may block thrombosis without eliciting major adverse bleeding effects (Mcbane et al., 2004). Current antithrombotic agents often cause a bleeding diathesis and the availability of antithrombic compounds that minimize the bleeding would be desirable for thrombosis treatment.

1.4.3.1 The role of Gas6 in protection against thrombosis

A recent study using gas6-null mice showed that gas6 plays an important role in thrombosis. Gas6 knockout mice display a platelet phenotype. These mice are protected against arterial and venous thrombosis, but do not suffer any bleeding. This phenomenon is partially due to defective platelet aggregation and secretion (Angelillo-Scherrer et al., 2001).In the absence of gas6, signaling by platelet aggregation by such agonists as ADP, collagen and thrombin was not completely blocked but produced smaller, loosely packed and incompletely degranulated platelet aggregates. Thus, secretion and aggregation of platelets in gas6 deficient mice could occur but was less efficient than in the wild type mice.

In platelets, gas6 is found in α-granules; it is secreted and binds to its receptor upon platelet activation. Since, gas6 null mice have normal expression of gas6 receptors, namely Axl, Mer and Rse, the platelet defects were not related to downregulation of these receptors indicating that absence of gas6 is responsible for this platelet defect (Angelillo-Scherrer et al., 2001). In contrast, a study by Balogh et al. (2005) failed to show the presence of gas6 in human platelets as demonstrated by highly sensitive antibody-based assays. The concentration of gas6 in human plasma was found to be 0.16 to 0.28 nM. Therefore, the previously proposed role of gas6 in platelet aggregation by Angelillo-Scherrer et al. (2001) may be due to gas6 secreted into the circulation. This circulating gas6 might be produced by endothelial cells, vascular smooth muscle cells and/or fibroblasts.

Furthermore, Gould et al. (2005) demonstrated that selective inhibition of either gas6 or any of Axl family of receptors (Axl, Mer and Rse) inhibits platelet degranulation

and aggregation. The magnitude of inhibition by each individual receptor was as significant as that of gas6. All three receptors are present on platelets; however, Axl appears to be the most abundant. Thus, based on these studies it can be concluded that gas6 plays significant role in protection against thrombosis.

In addition, a most recent study by Angellilo-Scherrer et al. (2005) demonstrated that each gas6 receptor (Rse, Axl, and Mer) is necessary for the platelet-stabilization effect of gas6 *in vitro*, whereas absence of any of these receptors protects mice against thrombosis *in vivo* as shown by receptor knockout studies. Thus, mice lacking any one of the three receptors have normal initial bleeding times and are resistant to thrombosis, but they do not suffer spontaneous bleeding. This effect is attributed to platelet dysfunction as demonstrated by platelet transfusion experiments. Moreover, deficiency of any of the gas6 receptors protected mice against thrombosis suggesting that each receptor has a comparably important role in platelet aggregation.

Remarkably, the cross-talk among receptors was observed in the form of tyrosine phosphorylation; in the absence of Axl, Rse, or Mer, gas6 was unable to induce tyrosine phosphorylation of the other two. This could be explained by the fact that during platelet activation, a substantial amount of gas6 receptors reach the platelet surface and a lack of any one of these receptors dramatically reduces the expression of the others resulting in reduced gas6 binding. These findings indicate that each gas6 receptor is regulated by expression of the others.

Moreover, phosphorylation of these receptors by gas6 is crucial in platelet function when platelets come into close contact with each other. Once activated by gas6, Axl activates PI3 kinase and Akt which play an important role in strengthening platelet

aggregation during the irreversible phase of aggregation (Kovacsovics et al., 1995; Trumel et al., 1999). Therefore, gas6 is a necessary amplification signal in pathologic conditions. Since the Gla domain of the vitamin K-dependant proteins is crucial in their blood coagulation activity and may also play significant role in regulation of cell activity, it is of great importance to examine its function, especially, as it relates to the gas6-Axl system.

1.5 Gamma-carboxylation

1.5.1 Gla domain function

Gas6 is the first γ-carboxyglutamic acid (Gla) domain-containing ligand that has been shown to regulate cell activity. Thus, it is important to analyse the biochemical role of the Gla domain in gas6. Gamma-carboxylation is a post-translational modification of several proteins including the procoagulant factors II, VII, IX and X, the anticoagulant proteins S and C, the recently described gas6, as well as two proteins found in bone, matrix Gla protein and osteocalcin. This enzymatic process adds a second carboxyl group to the gamma carbon of glutamic acid in the presence of the cofactor vitamin K. In the absence of vitamin K or in the presence of vitamin K antagonists, the synthesis of biologically active gamma-carboxylated proteins is impaired (Stenflo et al., 1972; Banchard et al., 1987).

The dietary source of vitamin K is found green leafy vegetables and it is also synthesized endogenously by the host's intestinal flora. The liver is the site of synthesis of the vitamin K dependant proteins associated with hemostasis (Shearer, 1995). No excess of vitamin K is present in the adult and low dietary intake of this essential vitamin

can produce sharp drops in its plasma levels in just few days. In the newborn, insufficient levels of vitamin K cannot fully carboxylate precursor proteins which may result in hemorrhagic disease of the newborn.

Vitamin K is recycled for re-use in the γ-carboxylation reaction. It starts of as a hydroquinone form of vitamin K which acts as a cofactor for the membrane bound vitamin K dependant carboxylase. This enzyme generates carboxyl groups on glutamic acid in the presence of CO_2 and O_2 resulting in a series of Gla residues on the N-terminus of the vitamin K-dependent proteins. Depending on the protein, there could be 9 to 12 Gla residues present on the N-terminus. This posttranslational modification renders the side chains of Gla negatively charged and capable of binding divalent cations such as calcium and magnesium (Saxena et al., 2001). The main function of the Gla domain is to allow Gla containing proteins to bind to phospholipid membranes. Upon binding, the Gla domain undergoes a change in its tertiary structure in which a series of hydrophobic residues become exposed to solvent. These residues constitute a phospholipid-binding site (Freedman et al., 1996). Once utilized, vitamin K is recycled from its epoxide form back to reduced vitamin K by the enzyme called the vitamin K_1 2,3-epoxide reductase (VKOR) (Cain et al., 1998). This cycle can be inhibited by coumarin anticoagulants (e.g. warfarin) by blocking the reduction of epoxide to its quinone form (Figure 1-3).

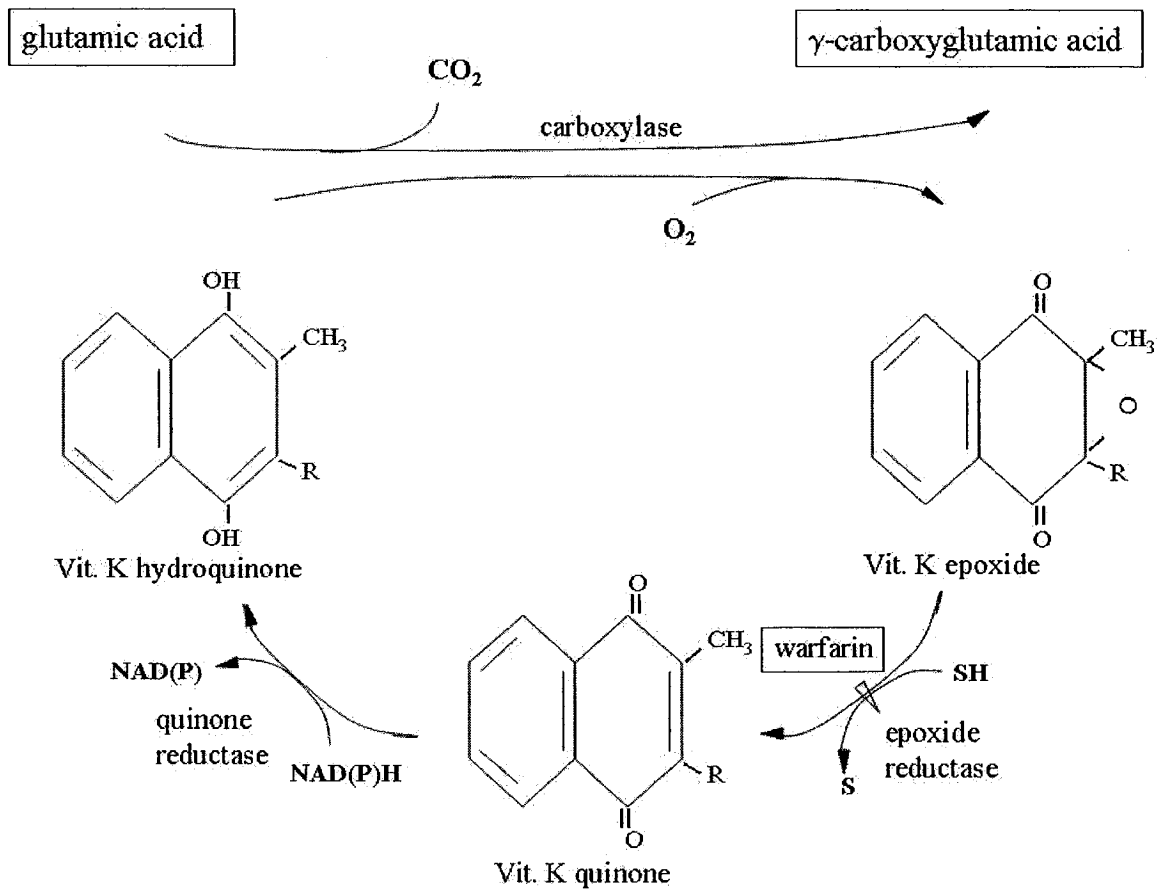


Figure 1-3. Vitamin K Cycle.

1.5.2 Importance of γ-carboxylation in gas6

Ever since gas6 was classified as vitamin K dependant protein there has been significant interest in the function of its Gla domain. As a vitamin K dependant ligand, gas6 can influence a variety of cellular functions such as cell growth (Crosier et al., 1997) and mitogenesis (Ling et al., 1995), cell transformation (Janssen et al., 1991), cell survival (Goruppi et al., 1996), and the clearance of apoptotic cells (Nakano et al., 1997). The Gla domain of gas6 and its carboxylation state may significantly affect these processes.

There is an apparent controversy in the literature about the importance of the Gla domain in gas6 function. Initial studies by Mark et al. demonstrated that receptor binding occurs through the C-terminal LG domain and that neither the Gla domain nor the EGF domains is essential to receptor activation. These experiments utilized gas6 in which Gla domain alone or Gla and EGF domains had been deleted. Moreover, while Gla and EGF domains were not necessary for receptor activation, they may contribute indirectly to this process. These domains may modulate gas6 activity *in vivo* by binding to membranes and phospholipids in a calcium dependant manner and establishing a local concentration gradient of the ligand, or by concentrating the ligand on the surface of receptor-bearing cells.

1.5.2.1 Gla domain of gas6 is necessary for its activity

It has been postulated that the Gla-rich N-terminus of the gas6 molecule is required to juxtapose the molecule to negatively charged phospholipid membranes. The first indication of the importance of the Gla domain in gas6 function came from the study of Nakano et al. (1996) where gas6 binding to cell surface was influenced by the calcium. This study shows that, in the presence of calcium, gas6 can bind to the cell surface whereas the binding is inhibited by addition of EDTA. Other studies have found that the Gla domain is necessary for maximal activity of gas6 (Tanabe et al., 1997). Tanabe et al. demonstrated that Gla domain of gas6 was necessary for both receptor binding and the mitogenic activity of this protein. Initial experiments, utilizing uncarboxylated gas6 (grown in the absence of vitamin K) and chimeric soluble receptor (Axl-Fc) composed of the extracellular ligand-binding domain of Axl fused to the Fc region of human immunoglobulin IgG1, showed that the C-terminal LG domain of gas6 was responsible for receptor binding (soluble Axl). Moreover, the binding ability to soluble Axl was significantly decreased in Gla-deficient gas6 as compared to gas6 with fully carboxylated Gla domain. Binding analysis revealed that Gla-deficient gas6 had a significantly lower binding affinity for soluble Axl (K_D of 4.1nM) as compared to fully carboxylated gas6 (K_D of 0.4nM) (Tanabe et al., 1997). Furthermore, this study also showed that Gla domain of gas6 was important in its mitogenic activity where carboxylated gas6 significantly stimulated the DNA synthesis of NIH3T3 cells, but Gla-deficient Gas6 (-Gla) did not.

1.5.2.2 Gla domain of gas6 promotes receptor binding

Early studies by Nakano et al. (1996) and Tanabe et al. (1997) demonstrate that the Gla domain of gas6 may have a promoting effect on the ligand-receptor interaction on the cell membrane. Other studies have also found that the Gla domain has a critical role in the functional properties of gas6, as supported by the following evidence: (i) the mitogenic effect of gas6 on VSMC is inhibited by warfarin (Nakano et al., 1997); (ii) gas6 binding to human osteosarcoma cell line is calcium dependant (Nakano et al., 1996); (iii) uncarboxylated rat gas6 cannot stimulate Axl phosphorylation in CHO cells (Tanabe et al., 1997); (iv) Gla domain is indispensable for the mitogenic activity of gas6 on mesangial cells (Yanagita et al., 2002; Yanagita et al., 1999); (v) splice variants of gas6 that can be proteolytically processed such as removal of the Gla domain, suggest possible regulatory role of Gla domain (Goruppi et al., 1997), and (vi) computer modeling of Gla domain of gas6 suggests that it could be capable of binding negatively charged phospholipid membranes (Perera et al., 1997).

On the other hand, findings of Mark et al. (1996) propose an alternative hypothesis where receptor binding is not affected by the state of the Gla domain. This study shows that a deletion variant of gas6 lacking the Gla domain bound and activated Axl and Sky receptors, suggesting that the receptor binding portion is found in the C-terminal of the gas6 molecule. This observation could suggest that the absence of a complete Gla domain fixes the conformation receptor binding site in its active form (Mark et al., 1996) and that the presence of decarboxylated "Gla domain " fixes the receptor binding site conformation in its inactive form. Conversely, in the presence of a

properly γ-carboxylated Gla domain, calcium can induce conformational change from an inactive to active form. Thus, the Gla domain of gas6 can act as a regulatory domain.

Taken together, these studies confirm that Gla domain of gas6 is important in its function.

1.5.2.3 Gla domain of gas6 is important in normal retinal function

To further study the role of the Gla domain of gas6, Hall et al. have examined its importance in normal retinal function. The receptor tyrosine kinase Mer has been shown to be important in this process. Gas6, a ligand for Mer, can stimulate the phagocytosis of the photoreceptor outer segment (OS) by normal rat retinal pigment epithelium (RPE) cultured cells (Hall and Abrams, 1987). One of the main functions of RPE is to phagocytose the shed tips of the photoreceptor outer segment (OS). Once this process is inhibited, death of the photoreceptor results and is accompanied by a progressive loss of vision.

Mer, a member of the Axl family of receptors tyrosine kinases, plays an important role in OS phagocytosis as demonstrated by Vollrath et al. and Feng et al. where a genetic defect in RCS rats, a model of defective OS phagocytosis, can be cured and OS phagocytosis restored upon transfection of full length of Mer mRNA in RPE cells *in vivo* and *in vitro*. A mutation in Mer has also been shown to cause blindness in humans (Thompson et al., 2002).

Phagocytosis of OS is dependant on presence of serum and gas6 is able to completely replace serum in this process (Hall et al., 2001). In their follow up study, Hall et al. were able to demonstrate that proper γ-carboxylation of gas6 in the presence of vitamin K and

calcium is crucial for gas6-mediated OS phagocytosis by cultured RPE cells and is dependant on gas6 binding to OS. Association of gas6 and OS is saturable and is dependant on Gla domain of gas6. Thus, gas6 binds to OS in a calcium dependant manner and that binding is obligatory for OS phagocytosis to occur. This binding is mediated through the Gla domain of gas6 and is analogous to other systems in which calcium allows the binding of gas6 to phosphatydylserine (PS).

1.5.2.4 Significance of γ-carboxylation of gas6 in glomerular disease

Gas6 and γ-carboxylation play a significant role in glomerular disease. Mesengial cell proliferation is the hallmark of glomerular disease and γ-carboxylation of gas6 may play an important role in its progression. Therefore, Yanagita et al. have studied whether warfarin, a γ-carboxylation inhibitor, can inhibit mouse mesangial cell proliferation by focusing on gas6. Warfarin has been used to treat glomerular diseases since the 1970s, and some trials have proved it is beneficial in improving the prognosis (Zimmerman et al., 1983; Kalowski et al., 1973; Woo et al., 1987; Woo et al., 1991). A putative target of warfarin is gas6.

1.5.2.4.1 Gla domain of gas6 and mesangial cells

In mesangial cells, gas6 acts as a mitogen as demonstrated by thymidine incorporation. This mitogenic effect is prevented by soluble AxlECD which captures recombinant gas6 and inhibits its binding to endogenous cell surface receptors, resulting in inhibition of receptor-dependent signal transduction (Yanagita et al., 1999). Furthermore, gas6 induces phosphorylation and subsequent ERK activation which is observed during cell proliferation. The proliferative effect of gas6 is significantly

inhibited by warfarin as is ERK activation. Taken together, these results indicate that γ-carboxylation is necessary for gas6 induced proliferation of mesangial cells.

In a follow up study, Yanagita et al. demonstrated that warfarin was able to significantly inhibit mesangial cell proliferation and therefore reduce the severity of glomerular injury *in vivo* presumably by altering gas6-Axl signaling. Treatment with warfarin reduced the expression of PDGF-B, a known growth factor that plays critical role in glomerulonephritis (GN), indicating that the Gas6/Axl pathway can affect platelet-derived growth factor-B (PDGF-B) production *in vivo* and plays significant role in GN. Expression of PDGF-B mRNA was induced in GN in the presence of gas6, whereas the induction was abolished when treated with warfarin. These findings suggest that the Gas6/Axl pathway modulates growth factor production, regulates mesangial cell proliferation and plays a critical role in GN that is dependent on proper γ-carboxylation of gas6 (Yanagita et al., 2001).

1.5.2.4.2 Role of STAT3 in mesangial cell proliferation

Yanagita et al. uncovered downstream targets of gas6-Axl interactions necessary for mesangial cell proliferation. A follow up study demonstrated that STAT3, a member of the STAT (signal transducers and activators of transcription) protein family, plays a significant role in gas6-mediated mesangial cell proliferation *in vitro* and *in vivo* (Yanagita *et al.*, 2001). The transforming activities of Eyk (Mer) depend on phosphorylation of STAT3. As well, constitutive activation of Eyk, a member of the Axl superfamily (Besser et al., 1999), results in cell transformation *in vitro*. The transforming activities of Eyk are dependent on STAT3 phosphorylation.

The STAT family of proteins are latent transcription factors activated by phosphorylation (Decker and Kovarik, 1999). As a result, STAT proteins dimerize and translocate to the nucleus (Fukada et al., 1996). Once in the nucleus, STAT proteins bind to DNA enhancer sequences of genes such as the immediate early growth response genes. Activation of STAT proteins has been implicated in differentiation and growth regulation.

Yanagita et al. have demonstrated that gas6 phosphorylates STAT3 and that this phosphorylation is dependent on Axl. Following phosphorylation, STAT3 translocates to the nucleus where it induces STAT3 dependent transcription. This gas6 induced STAT3 activation then triggers mesangial cell proliferation.

In an *in vivo* model of glomerulonephritis, STAT3 is heavily phosphorylated in the nuclei of glomerular cells that parallels the increase in mesangial cell proliferation and gas6 expression. This effect is inhibited by warfarin which underlies the importance of the Gla domain of gas6 in these processes. Treatment with warfarin completely inhibited the phosphorylation of STAT3 as well as mesangial cell proliferation

1.5.2.4.3 Link between γ-carboxylation and glomerular hypertrophy

To further study the link between γ-carboxylation of gas6 and glomerular disease, Nagai et al. examined the possibility that gas6/Axl interactions can contribute to the pathogenesis of diabetic glomerular hypertrophy.

Diabetes is the most common cause of end stage renal disease and glomerular hypertrophy is a hallmark in the early phase of diabetic nephropathy. There are several characteristics of diabetic nephropathy such as: persistent albuminuria and mesangial

expansion followed by glomerulosclerosis and a decline in renal function (Nagai. et al., 2003). Several factors such as transforming growth factor-β (TGF-β) and angiotensin II have been implicated in the development of diabetic nephropathy (Lehmann et al., 2000). Angiotensin-converting enzyme inhibitors and/or type I angiotensin receptor blockers have been proven to be effective to some extent and they decrease the risk of developing nephropathy by only 12.5% in type 2 diabetic patients (Ravid et al., 1998). Thus, novel more efficient therapies are necessary for patients with diabetic nephropathy.

Recently, this group has demonstrated that gas6 acts as an autocrine growth factor for mesangial cells and as such plays a significant role in mesangial cell proliferation. Furthermore, gas6 and its receptor Axl play a crucial role in the development of glomerulonephritis since uncarboxylated gas6 inhibits mesangial cell proliferation by specific blockade of the gas6-mediated pathway in a mesangial proliferative model of glomerulonephritis (Yanagita et al., 2001 and 2002).

Nagai et al. have utilized the streptozotocin (STZ) induced diabetic rats and mice to examine the role of gas6/Axl in the early phase of diabetic nephropathy *in vivo*. They have analyzed the glomerulus after 12 weeks of STZ injection and found that expression of both gas6 and Axl was significantly increased in the STZ-treated group and that they were mostly localized to endothelial and mesangial cells. Since the gas6 and Axl expression was induced in these diabetic mice, modulation of this pathway could play a role in the development of diabetic nephropathy in the early phase of the disease process. More interesting is the finding that the expression of Axl was significantly inhibited in warfarin treated rats as compared to untreated rats. Furthermore, in warfarin treated rats, the MAP kinase pathway was inhibited due to lack of phosphorylation. Thus, warfarin

treatment showed beneficial effect on mesangial and glomerular hyperthrophy by preventing an increase of mesangial and glomelural proliferation as well as improving hyperfiltration and excretion of urinary albumin.

To confirm the specificity of warfarin on the gas6-Axl pathway, Nagai et al. examined STZ-treated gas6 knockout mice. Preliminary results demonstrated that mesangial cell and glomerular surface areas in diabetic wild type mice were significantly larger than those in wild type untreated mice. However, in diabetic Gas6 knockout mice, the increase of both areas was significantly suppressed. These data indicate that gas6 is involved in the development of the initial phase of diabetic nephropathy and suggest that warfarin inhibits diabetic nephropathy specifically through the gas6-mediated pathway. Similarly, only carboxylated gas6 (100 ng/ml) was able to induced mesangial cell hyperthropy *in vivo* whereas this effect was inhibited in the presence of uncarboxylated gas6 and soluble Axl ECD. Taken together, these results indicate that gas6 can induce mesangial cell hypertrophy, characteristic of the early stage of diabetic nephropathy, and that warfarin is effective in preventing the progression of diabetic nephropathy. These effects of gas6 are not only dependant on its proper carboxylation but also on its interaction with the Axl family of receptor tyrosine kinases.

1.6 Axl Family of Receptor Tyrosine Kinases

Receptor tyrosine kinases belong to class of proteins that transduce signals from the extracellular region to the cytoplasm, ultimately controlling cell proliferation and differentiation. These receptors share a similar structure and are composed of an extracellular ligand-binding domain, followed by a transmembrane domain and then a catalytic kinase domain. Upon ligand binding, the extracellular domain receptor oligomerizes resulting in phosphorylation and activation of the kinase domain. In addition to their catalytic function, the intracellular domains of receptor tyrosine kinases also serve as binding sites of other components of the signal transduction pathway. Some proteins contain src homology 2 domains (SH2) that bind in a sequence–specific manner to phosphorylated receptor tyrosine kinases (Cantley et al., 1991; Koch et al., 1991; Songyang et al., 1993). Axl, Mer and Rse are three members that belong to this family of receptor tyrosine kinases.

1.6.1 Axl (Ark, Ufo, Tyro7)

In 1991, Axl was isolated as a 140 kDa transforming gene from DNA of patients with chronic myelogenous leukemia (O'Brian et al., 1991). It is a member of a family of receptor tyrosine kinases that include two other receptors, c-Mer and Rse. Axl is a type I transmembrane receptor whose extracellular portion is composed of two immunoglobulin-like domains (IgL) and two fibronectin type III domains (FN III). It also has a short cytoplasmic tail that has tyrosine kinase activity.

The kinase domain of Axl contains two PI3 kinase consensus binding sites suggesting that Axl may complex with PI3 kinase. Although similar to other receptors,

Axl represents a novel subclass of tyrosine kinases based on the unique structure of an extracellular region consisting of two immunoglobulin-like domains and two fibrinectin-like (FN) type III domains. The two FN type-III motives lie between amino acids 224 and 448 and were first identified as 60-100 amino acid tandem repeats reiterated 15 times in fibronectin (Skorstengaard et al., 1986).

A number of extracellular matrix proteins and receptors contain FN type III repeats including neuronal adhesion molecules, the growth horomone/receptor prolastin family, several tyrosine phosphatases and the insulin receptor family of receptor tyrosine kinases (Bates, 1987; Harrelson and Goodman, 1988; Norton et al., 1988; Patthy, 1990) Thus, based on the homology in the extracellular and kinase domains, Axl appears most closely related to the insulin family of receptor tyrosine kinases. Axl expression is seen in most human cell lines including cell lines of epithelial, mesenchymal, and hematopoietic origin (Table 1-1).

TABLE 2. Summary of *axl* expression

Sample	Cell line	Expression[a]	
		Northern analysis	PCR analysis
Nude mouse tumor cell lines	AF6295[b]	++++	++++
	AF3642[b]	++++	++++
Hematopoietic cell lines			
Lymphoid	LAM	ND	−
	DHL-4	−	−
Myeloid			
Promyelocytic	HL60	−	−[c]
Acute myelogenous leukemia	SKL1	−	++
CML blast phase	KOPM-28	−	++
	EM2	−	+
	EM3	−	−
	K562	−	++
Primary hematopoietic tissues	NPB 1[d]	ND	−
	NPB 2	−	+
	CML/CP[e]	−	+
	MDS 1[f]	−	−
	MDS 2	−	+
Breast cell lines	MDA-157	−	−
	MDA-468	−	+++
	SK-BR3	−	−
	BT-20	−	++
	BT-474	−	+
	MCF-7	−	+
	600 PEI	−	++
Normal breast epithelia	337	−	++
Miscellaneous			
Cervical cancer	HeLa	+++	+++
Lung cancer	A549	+++	+++
Epidermoid cancer	A431	+++	+++
Normal human fibroblast cell lines	WI 38	−	+++
	BG-9	+++	+++
	IMR-90	+++	+++
Mouse fibroblast cell line	NIH 3T3	+	+++[g]

[a] Semiquantitated as follows: ++++, very high; +++, high; ++, moderate; +, weak; −, undetectable. ND, not determined.

[b] Derived from secondary nude mouse tumors arising from transfection of DNA from a patient with blast crisis CML (AF6295) or a patient with chronic-phase CML (AF3642).

[c] The fragment detected was approximately 600 bp. The sequence of this fragment was distinct from that of the *axl* protein and may represent a related kinase.

[d] RNA was extracted from normal peripheral blood leukocytes.

[e] RNA was extracted from peripheral leukocytes from a patient with chronic-phase CML.

[f] RNA was extracted from peripheral leukocytes from a patient with myelodysplasia.

[g] Analysis was done with use of only *axl* primers specific for the kinase domain.

Table 1-1. Summary of Axl expression (O'Brian et al., 1991)

In addition Axl is expressed in normal, non-transformed cells including primary fibroblasts, breast epithelium, and endothelial cells. Furthermore, overexpression of Axl is required for manifestation of a transforming phenotype and low-level expression is insufficient for neoplastic transformation (O'Brian et al., 1991). Axl also contains a differentially spliced region that can produce a distinct variant of this protein. However, the function of this differentially spliced protein remains unclear.

Due to its unique structure, Axl has been implicated in variety of cellular functions. First, the transforming activity of Axl plays a role as a proto-oncogene in proliferation (Sainaghi et al., 2005) Second, Axl expression is detected in a myriad of tissues where it plays a role in normal cellular activity (Goruppi et al., 1997). Third, Axl expression is altered in hematopoietic differentiation. Axl levels are augmented in patients with chronic myologenic leukemia (Janssen et al., 1991; O'Bryan et al., 1991; Neubauer et al., 1994).

The role of Axl is defined by its interaction with its only ligand, gas6. Axl was first linked to its receptor gas6 by Varnum et al. when it was isolated from media of the human fibroblast cell line, Hs27, by immunoaffinity chromatography using Axl as bait. This study also noted that Axl expressing cell lines, Wi38 (human lung fibroblast cell line) and Hs27, have greatly elevated phosphotyrosine content of Axl. This phosphorylation was blocked by soluble Axl demonstrating that Axl activity is due to direct binding to its ligand, gas6, rather than by an indirect mechanism (Varnum et al., 1995).

Furthermore, in CHO (Chinese hamster ovary) cells, Axl phosphorylation was detectable when Axl was stimulated with gas6 at concentrations as low as 3ng/ml, with a

maximal response obtained at 100 ng/ml of gas6. This binding was demonstrated by chemical cross-linking at 1nM, and phosphorylation at subnanomolar concentrations, demonstrating that gas6 stimulates Axl at a concentration expected for a ligand. Gas6 binds to Axl with a dissociation constant (K_D) of 4nM (Varnum et al., 1995).

1.6.2 Rse (Tyro3, Dtk, Etk, Brt, Tif, Sky)

Rse was first isolated from a chicken proto-oncogene originally described as a retroviral transforming gene (Jia and Hanafusa, 1994). Rse cDNA was later isolated from human and murine brain tissue (Mark et al., 1993). Murine and human Rse share 90% homology, with a sequence identity of 85% in the extracellular region and 93% in the intracellular domain.

In the kinase domain, human Rse is most similar to the human receptor tyrosine kinase Axl, 64% homology (Mark et al., 1994). Since human Rse and Axl share similar structural organization with respect to the extracellular domain, Rse can be placed in the Axl family of receptor tyrosine kinases. Similar to Axl, Rse runs on SDS-PAGE as a doublet at 120 and 140 kDa corresponding to two different glycosylated isoforms. Rse mRNA is expressed in a variety of tissues with highest expression in the brain and the kidney and lower expression in the heart, placenta, liver lung, skeletal muscle and pancreas. Rse is also expressed in smaller quantities in breast, adrenal gland, and small and large intestines. In addition, the Rse mRNA can be detected in several carcinoma cell lines (Mark et al., 1994).

In addition to Axl, Rse is also a receptor for gas6 and its affinity for gas6 is much lower than that of Axl (Stitt et al., 1995). In CHO cells overexpressing Rse, gas6 was able to induce phosphorylation of this tyrosine kinase receptor. Furthermore, this

phosphorylation was abrogated in the presence of soluble Rse protein indicating that Rse phosphorylation was most likely induced by direct interaction with its ligand, gas6, and not by indirect phosphorylation of Rse by other receptor tyrosine kinases (Ohashi et al., 1995).Rse phosphorylation in response to gas6 is dose dependant with half-maximal stimulation at 1nM and minimal detectable phosphorylation at 0.5nM. This is comparable to a K_D (0.3nM) of binding of gas6 to membranes of vascular smooth muscle cells (Nakano et al., 1995). Gas6-Rse interactions were reported to stimulate bone resorption (Katagiriet al., 2001).

1.6.3 Mer (c-mer, Nyk, Eyk)

Mer was originally isolated by screening a human B-lymphoblastoid lambda gt11 expression library using anti-phosphotyrosine antibodies yielding complementary DNAs encoding active tyrosine kinases. The results were used to obtain the sequence of a novel 984 amino acid transmembrane tyrosine kinase. Analysis of the complementary DNA revealed that this 984 amino acid receptor tyrosine kinase structurally resembled the Axl family of tyrosine kinases since it is composed of extracellular immunoglobulin and fibronectin type III domains followed by the cytoplasmic kinase domain (Jia and Hanafusa, 1994). The novel tyrosine kinase, c-Mer, was not expressed in normal B- and T-lymphocytes but, unlike Axl, was expressed in numerous neoplastic B- and T-cell lines. Transcripts of this receptor tyrosine kinase were also detected in normal peripheral blood monocytes and bone marrow (Graham et al., 1994).

Mouse homologue of Mer reveals an overall identity of 88% with human Mer. This mouse homologue is expressed in many tissues and has a unique expression pattern among the Axl family members. In normal adult hematopoietic cells, Mer is

predominantly expressed in the monocytic lineage. Mouse c-Mer is expressed during most stages of embryological development beginning in the morula and blastocyst and progressing through the yolk sac and fetal liver stages suggesting a role in the developing mouse (Graham et al., 1995). It was also independently isolated as a receptor tyrosine kinase potentially involved in the development of glioblastomas, and called Nyk (for NCAM-related tyrosine kinase; Ling and Kung, 1995). The transforming gene of the avian retrovirus RPL30, v-eyk, is derived from the chicken homologue of Mer, c-eyk (Jia and Hanafusa, 1994).

In normal human tissues, Mer mRNA is preferentially expressed in the adult brain and at lower levels in kidney, ovary, prostate, lung and testis (Mark et al., 1994; Graham et al., 1994). Similar to Axl and Rse, Mer also acts as a receptor for gas6 (Chen et al., 1997). The dissociation rate of gas6 from Mer is very rapid as compared to Axl. Consequently, the equilibrium dissociation constant (K_D) of gas6 for Mer was higher than that observed for Axl, suggesting that gas6 has highest affinity for the Axl receptor (Nagata et al. 1996).

In addition, gas6-Mer interactions play role in MAP kinase pathway activation (Chen J et al., 1997). It promotes testicular cell survival (Chan et al. 2000), induces the survival of human oligodendrocytes via a phosphatidylinositol 3-kinase-dependent pathway (Shankar et al., 2003) and participates in platelet function (Chen et al., 2004). Mer has also been found to be upregulated in prostate cancer and thus, may have a potential role in prostate cancer progression (Wu et al. 2004).

1.6.4 Receptor knockout studies

Receptor knockout studies provide significant insight in the role of Axl tyrosine kinase receptor family. Recently, murine knockout data have shed some light on the importance of the Axl receptor tyrosine kinase family. Homozygous null mice for any single receptor do not produce any apparent clinical phenotype (Lu et al., 1999). These results indicate that no single receptor is essential during embryonic development. However, the triple receptor null mouse displays multiple major organ defects, neurological abnormalities and physiological deficits. A variety of adult tissues such as brain, prostate and blood vessel walls showed altered histology, increased cellular degeneration and apoptosis. Furthermore, these mice are blind and have grossly enlarged spleens. These results demonstrate that this family of receptors is essential for the trophic maintenance and homeostatic balance of a wide variety of cell types in mature mammalian tissues (Lu et al., 1999).

The most severe phenotypes is observed in the adult male gonads where mice are infertile due to failure of Sertoli cells to provide trophic support for germ cells, suggesting a role of these receptors in reproductive development. Another phenotype has also been described by this group, one in which triple receptor null mice demonstrate immune dysregulation characterized by autoimmunity and lymphoproliferative disorders (Lu and Lemke, 2001). Triple mutant females show recurrent thromboses and hemorrhage in several tissues, including the brain. These abnormalities are associated with the presence of antibodies to phospholipids and autoimmune syndromes. Furthermore, these mice exhibit abnormally high levels of circulating antibodies to double stranded (ds) DNA.

In general, mice carrying any two of three genes deleted exhibit higher levels of ds DNA antibodies than do single mutants. However, the triple-null mutant has the highest levels of circulating dsDNA antibodies (Lu and Lemke, 2001). The presence of various autoantibodies to collagen is also found in these mice. This is frequently detected in sera of patients with rheumatoid arthritis (Stuart et al., 1984). High levels of autoantibodies against phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol are also observed in the patients with RA.

Furthermore, most of the triple knockout females are unable to carry a pregnancy to term. However, it is important to note that the Tyro 3/Axl/Mer triple mutants survive throughout embryogenesis and exhibit no obvious developmental defects in any tissue. Remarkably, these triple knockout mice are indistinguishable from wild-type animals for the first two to three weeks after birth (Lu and Lemke, 2001). This lack of phenotype during the first weeks of development in triple knockout mice is unique to the Axl family. Thus, the phenotypes that develop in these mutants in immune, nervous, vascular and reproductive systems represent late-onset disorders and develop in more or less fully differentiated tissues and organs (Lu and Lemke, 2001; Lu et al., 1999).

As previously mentioned, the phenotypes of these knockouts are initially observed at the onset of sexual maturation at four weeks of age. These mice develop blindness due to photoreceptor degeneration, infertility as a result of degeneration of germ cells in the testes and ovaries at four to eight weeks of age, and more defective synaptic function than single knockouts making them prone to violent seizures.

Eventually their brains exhibit extensive neuronal loss and a pronounced spongiform encephalopathy. Most aged triple knockout mice are paralyzed (Lu et al., 1999).

More pronounced phenotypes are seen in Mer knockouts where mice are blind as a result of a complete postnatal degeneration of photoreceptors (Scott et al., 2001; Lu et al., 2001). Recently, studies have shown that in the animal model of retinitis pigmentosa, there is a 409 bp deletion (resulting in a frameshift and translation termination) in the rat Mer gene (D'Cruz et al., 2000) and that mutations in the human Mer gene have been detected in patients with retinitis pigmentosa (Gal et al., 2000). These Mer knockouts also display a delayed clearance of apoptotic cells, enhanced sensitivity to endotoxic shock (Camenisch et al., 1999) and a tendency to development of autoimmunity (Cohen et al., 2002; Scott et al., 2001). Also, Rse single mutants as young adults exhibit compromised central nervous system (CNS) function (diminished hippocampal long-term potentiation [LTP]) and neural degeneration accompanied by seizures and hind limb paralysis (Lu et al., 1999). Additional mice knockout studies of these receptors show that they may also play a significant role in protection against thrombosis.

1.6.4.1 Knockout mice protected against thrombosis

A recently characterized c-Mer null mouse demonstrates a platelet signaling defect that protects these mice against lethal venous thromboembolisam (Chen et al., 2004).Similar to gas6 knockout mice, Mer null mice exhibit an impaired platelet aggregation response and show protection from thrombosis. However, the antithrombotic response is less pronounced than in gas6 null mice. Another recent study demonstrated that loss of any one member of Axl receptor tyrosine kinase family protects mice against

life-threatening thrombosis (Angellilo-Sherrer et al., 2005). In addition, each of the gas6 receptors is necessary to transmit the platelet stabilization effect of gas6 in vitro. The deficiency of any one of the gas6 receptors protects mice against thrombosis to the same significant extent, suggesting that each of these receptors have an important role in platelet aggregation. However, the absence of any one of these receptors prevents tyrosine phosphorylation of the other two in the presence of gas6. These interesting findings suggest that a substantial amount of gas6 receptor reach the platelet surface only during platelet activation and that the lack of any one of these receptors significantly reduces the expression of other two receptors. This in turn, reduces binding of gas6 to its receptor and subsequently affects receptor phosphorylation (Angellilo-Sherrer et al., 2005). In addition to their role in protection against thrombosis, the Axl family of receptors, being tyrosine kinase receptors, plays a significant role in cell signaling.

1.6.5 Role of Axl family of receptors in cell signaling

In order to further understand the role of Axl, early studies of its signaling pathways have relied on chimeric receptors. In 32D (myloid progenitor) cells, chimeric receptor studies by Fridell et al. demonstrated that a chimeric receptor containing the extracellular domain of EGF receptor and the kinase domain of the Axl receptor was able to activate MAP kinase in a ligand dependant fashion. Conversely, full length Axl receptor did not activate MAP kinase in these cells in response to gas6. To clarify these conflicting results, Goruppi et al. have demonstrated, using NIH 3T3 fibroblast that express full length receptor, that Axl is able to induce MAP kinase activation in response to its ligand gas6. Further studies have implicated that Axl plays an important role in cell

survival, mainly through activation of PI3 kinase, a known antiapoptotic signaling molecule.

1.6.5.1 Axl activates PI3 kinase

Tyrosine kinase receptors are transmembrane proteins composed of an extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic domain harboring the tyrosine kinase activity (Ullrich and Schlessinger, 1990). Activation of receptor tyrosine kinases is induced by binding of ligands to the extracellular domain of these receptors with subsequent dimerization and autophosphorylation of tyrosines in the cytoplasmic domain. These activated phosphotyrosine residues then serve as docking sites for proteins that contain the *src* homology 2 domain (SH2) motif, such as PI3 kinase (Pawson and Schlessinger, 1993; Pawson and Gish, 1992).

The binding specificity of an SH2 domain containing protein is determined by the specificity of three amino acids C-terminal to the phosphorylated tyrosine of the receptor. Braunger et al. have identified that several of these SH2 domain proteins are substrates for the phosphorylated receptor tyrosine kinase Axl using human fetal liver and a human foreskin fibroblast cDNA expression library. Competition studies have allowed them to map out specific SH2 binding residues by tyrosine phosphorylated synthetic peptides. The most significant residue is found to be tyrosine 821(pY821) which, when inhibited, prevented binding of the p85α, p85β subunit of the SH2 domain of PI3 kinase and subsequent propagation of survival signal. Thus, this residue represents a flexible, multi-substrate docking site for the p85 subunits of PI3 kinase. Since pY821 represents a multi-

substrate binding site, there must be competition amongst substrates which is most likely dependant on local concentration and affinity of the substrate for Axl.

1.6.6 Role of Axl family of receptors in immune response

Not only is the Axl family of receptors important in cell survival but they also serve as an important regulator in the activation state of macrophages. Macrophages, dendritic cells and other antigen-presenting cells (APCs) are essential for antigen presentation to T and B regulatory cells and are crucial for an immune response. When activated, they produce cytokines that engage all cells of the immune system. However, when in an unchecked inflammatory environment, APCs can greatly damage host cells and tissues. Thus, the balance between the positive signals that activate APCs and the negative signals that inhibit them is indispensable for normal function of immune system. Disruption of this balance results either in an ineffective immune response, or, alternatively, a hyperactive one (Freitas and Rocha, 2000; Hanada and Yoshimura, 2002).

Engineered mouse mutants have provided evidence that the Axl family of receptor tyrosine kinases (RTKs) function as homeostatic regulators of APC activation. All three receptors of Axl family are found on the surface of monocytes and their derivatives; Mer is found in peripheral blood and bone marrow mononuclear cells, monocytes and macrophages but not in granulocytes and peripheral T and B lymphocytes (Graham et al., 1994 and 1995). Similarly, Axl is expressed by peripheral monocytes and macrophages, but not by granulocytes or lymphocytes (Neubauer et al., 1994 and 1997). Expression of all three receptors is elevated in regions enriched by macrophages and absent from regions enriched by lymphocytes (Lu and Lemke 2001).

1.6.6.1 Mechanism of action of Axl RTKs in immune response

The mechanism of action of macrophage regulation by the Axl family of receptors is as follows; (i) following the initial immune response such as bacterial infection, the Axl family of receptors induce signalling events that deliver an inhibitory signal to APCs, downregulating the expression of inflammatory cytokines and cell surface proteins associated with antigen presentation, (ii) once activated by the ligand, they the bind to apoptotic cells and play a role in their and phagocytosis by mobilizing these cells to the macrophages for phagocytosis (Scott et al., 2001; Cohen et al., 2002).

In the Axl family of null mice, both of the reactions described above are interrupted at the point at which APCs are maximally activated. Furthermore, triple Axl family knockouts can contribute to the development of autoimmune disease by failing to phagocytose and clear apoptotic cells, thereby presenting a host with autoantigens.

1.6.6.2 Triple knockout phenotype

A dramatic immune phenotype is observed in the triple receptor knockout. These mice display peripheral lymphoid organs and spleen of normal size and weight at birth, whereas, by six months of age, these organs become severely enlarged. By one year of age, the average spleen weight is ten times that of wild type mice. Lu et al. have shown that each of three receptors contribute to this lymphoid enlargement, with the Mer mutation exhibiting the greatest effect. This phenotype is due to aberrant proliferation of B and T lymphocytes that result in saturation of the peripheral lymphoid compartments leading to the presence of colonies of invasive lymphocytes in essentially every tissue

and organ of the body (Lu and Lemke 2001). These constitutively active lymphocytes lead to autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (Scott et al., 2001; Lu et al., 1999). Thus, the triple knockout of the Axl family of receptors results in a severely impaired immune system phenotype.

More specifically, macrophages from Mer knockout mice are slow to clear dead cells when presented with a large number of experimentally induced apoptotic thymocytes (Scott et al., 2001; Cohen et al., 2002). This delayed clearance of apoptotic cells is of great interest since gas6, a ligand for Axl family of receptors, can potentially interact with phosphatidylserine (PS) via its amino-terminal Gla domain (Nakano et al., 1997). During apoptosis, PS is exposed on the outer leaflet of the plasma membrane and allows the binding of the Gla domain of gas6, which may in turn bind to the Axl family receptor tyrosine kinases, thereby providing a mechanism for the direct mobilization of apoptotic cells to the macrophages that subsequently engulf them This phagocytosis is dependant on the kinase activity of the Axl family of receptors (Scott et al., 2001; Cohen et al., 2002).

Taken together, these findings suggest an important role of the Axl family of receptor tyrosine kinases in the immune response. Moreover, these receptors and their ligand are key players in homeostatic regulation of APC activation. Signalling events induced by gas6 interaction with the Axl family of receptors are of particular interest in various systems such as immune, vascular, reproductive, and nervous system since they can contribute to the development of human diseases.

1.7 Gas6/Axl

Gas6 was identified as a ligand for the receptor tyrosine kinase (RTK) Axl (Varnum et al., 1995). Although gas6 can bind all three receptors, its greatest affinity is for Axl (10nM). Gas6-Axl interactions have been examined in many different cell systems including vascular smooth muscle, the kidney, platelets, the central nervous system, bone and the eye.

1.7.1 Gas6-Axl interactions in NIH 3T3 fibroblasts

Initial studies of gas6-Axl interactions have used Axl expressing NIH 3T3 fibroblasts as a model system. In order to elucidate biological role of gas6 in the context of growth arrest, gas6 was assayed for its ability to induce cell division in arrested NIH 3T3 fibroblasts (Goruppi et al., 1996). This study demonstrated that gas6 was able to induce mitogenic activity only in high concentrations (400ng/ml) and stimulate entry in S phase in 50-60% of cells. Furthermore, gas6 induced cyclin D expression at low levels in serum starved NIH 3T3 fibroblasts after 3 hours whereas, cyclin A expression appeared only after 9-12 hours. This response was mediated by Axl as it is inhibited by the soluble extracellular domain of Axl (Goruppi et al., 1996). In addition, Axl induced MAPK activation consistent with the mitogenic activity of gas6. Moreover, gas6 acts as a survival factor since it protects serum starved NIH 3T3 fibroblasts from apoptosis. Thus, the initial Goruppi studies demonstrated two activities of gas6 in NIH 3T3 fibroblasts, mitogenic and survival.

1.7.1.1 Initial insights into gas6-Axl signaling

The study of gas6-Axl interaction in NIH 3T3 fibroblasts provides insight into subsequent signal transduction events. Gas6 mediates its survival through activation of PI3 kinase. Requirement for PI3 kinase in gas6 protection from apoptosis was demonstrated using the PI3K inhibitor wortmannin and dominant negative p85 subunit constructs. These latter constructs are unable to bind the p110 catalytic subunit of PI3 kinase (p85D110) in serum-starved fibroblasts and induce subsequent signaling events (Goruppi et al., 1997). In addition, gas6 activated not only PI3 kinase but also downstream molecular targets of PI3 kinase, namely, S6K, Src and Akt. The activation of S6K was inhibited by rapamycin, an S6K inhibitor. S6K inhibition completely abolished gas6 induced cell cycle reentry suggesting that gas6 requires activation of S6K to induce entry into the S phase (Goruppi et al., 1997). Moreover, both S6K and Akt activation are PI3 kinse dependant since wortamnnin, an irreversible PI3 kinase inhibitor, blocks their activity.

Furthermore, Goruppi et al. have demonstrated that Src, a protein tyrosine kinase important in cell differentiation, motility, proliferation, and survival (Thomas and Brugge, 1997), is involved in gas6-induced signaling but could not be co-immunoprecipitated with the Axl receptor (Goruppi et al., 1997). Gas6 addition stimulated the activation of endogenous Src even though the Axl cytoplasmic region lacks any known Src interacting consensus sequences (O'Bryan et al., 1991). NIH 3T3 fibroblasts stably expressing dominant negative kinase-inactive Src were unresponsive to the gas6 mitogenic effect, thus supporting Src involvement in gas6 signaling. These results are reminiscent of EGF induction of DNA synthesis, whereby the Src family of

tyrosine kinases is required even though interaction with the activated receptor was not observed in a reconstituted system (Roche et al., 1995).

To further characterize the signal transduction pathway by which gas6 protects NIH 3T3 fibroblasts from serum starvation-induced apoptosis, several well known antiapoptotic markers have been studied. It has been reported, as analyzed by dominant negative constructs, that gas6 requires the activity of the Ras-related small GTPases of the Rho family, namely, Rac. This was confirmed by complete abrogation of gas6-induced survival through overexpression of dominant negative Pak, a downstream target of Rac (Zhang et al., 1995). Rho GTPases perform important functions in controlling gene transcription, cell cycle control and apoptosis and are activated in response to proinflammatory cytokines, cellular stress and growth factors. This signaling pathway leads to stimulation of stress activated protein kinases c-Jun and p38 Map kinase which subsequently activate specific transcriptional factors (Coso et al., 1995; Minden and Karin, 1997; Robinson and Cobb, 1997). Ras is implicated in both induction and protection against apoptosis (Downward, 1988). Blocking Ras activity in gas6 treated NIH 3T3 abolished only the mitogenic, but not the antiapoptotic, activity of gas6 (Goruppi et al., 1999).

Active Akt is necessary for gas6 mediated protection from apoptosis (Goruppi et al., 1999). Once activated by gas6, Akt can induce Bad phosphorylation, a proapoptotic bcl-2 family member, and inactivate its proapoptotic signal in NIH 3T3 fibroblasts. Moreover, gas6 is able to induce ERK, JNK/SAPK and p38 kinase activation, all of which play a role in protection from apoptosis. In summary, gas6 interferes with cell death induced by growth factor depletion through activation of the PI3 kinase and Akt

pathway, resulting in the inactivation of the apoptosis-related protein Bad and the downregulation of activity of the apoptosis-related p38 MAP kinase.

1.7.2 Gas6-Axl in vascular system

Following initial studies in NIH 3T3 fibroblasts , many groups have shifted their research to more biologically relevant systems. A role of Axl in vascular physiology has been suggested by Nakano et al. This study reported that Axl activation in response to its ligand gas6 induced DNA synthesis in vascular smooth muscle cells (VSMC) when administered with Angiotensisn II, thrombin and lysophosphatidic acid (Nakano et al., 1995). VSMC proliferation and migration is triggered by vascular injury and is a response to a dynamic and ordered expression of receptors and growth factors. The most common model used to study the molecular response to vascular injury is rat carotid balloon injury. Studies based on this model suggest that activation of receptor tyrosine kinases resulting in subsequent intracellular signaling transduction is a critical event in VSMC proliferation and migration. There is a significant increase in expression of growth factors such as basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) in injured rat blood vessels (Schwartz et al., 1995).

Melgrano et al. have demonstrated that gas6 and Axl expression are increased and localized to cells of the neointima after balloon injury. Axl mRNA and protein expression is increased by Angiotensin II (Ang II) and thrombin suggesting important interactions of both Ang II and thrombin with Axl. Furthermore, this study shows that Ang II is also an important regulator of Axl expression in vivo. In addition, the study demonstrates that an increase in Axl mRNA expression is observed at 7 and 14 day post vessel injury and that

the protein resides in both the vascular media and neointima at 14 days. Moreover, gas6 mRNA expression is increased between 6 hours and 3 days after the injury with continued elevation for up to 4 weeks. Thus, taken together these results underscore the importance of gas6 and Axl in vascular injury and establishes that expression of both proteins is increased during rat carotid injury with a time course paralleling neointima formation.

1.7.2.1 Role of gas6-Axl in cell migration

To further study the importance of gas6-Axl in the vascular system, several groups have examined the role of this interaction in vascular cell migration. Gas6-Axl interactions have been shown to be involved in direct VSMC migration as demonstrated by an in vitro chemotaxis assay (Fridell et al., 1998). Directed migration of VSMC to the intima and subsequent proliferation of the neointima are events involved in atherogenesis following vascular injury (Bornfeldt et al., 1995). As a cofactor for proliferation of rat VSMC, gas6 could also act as a potential chemotactic agent for these cells. Thus, Fridell et al. have demonstrated that human recombinant gas6 was able to induce chemotaxis of rat VSMC reaching maximal chemotaxis at 200ng/ml of gas6. Similarly, rat VSMC proliferation and Axl autophosphorylation also reached maximal levels at this gas6 concentration. This effect was attenuated by the addition of the soluble, extracellular domain protein Axl (Axl-ECD).

In a similar fashion, human aortic smooth muscle cells (AoSMC) migrated in response to recombinant human gas6. Gas6-induced chemotaxis of AoSMC was also reduced in the presence of purified Axl-ECD. Furthermore, gas6 was able to induce

proliferation of these cells as measured by a DNA synthesis assay. These effects are mediated by Axl in response to gas6. In cells overexpressing Axl, migration was significantly increased (5 fold) when compared to wild type cells confirming previous findings that Axl mediates gas6 induced migration of VSMC. Based on these findings, Fridell et al. have proposed the following hypothesis for gas6-Axl involvement following the vascular injury: an increase in local gas6 concentration induces migration of medial smooth muscle cells to the intima. Subsequently, Axl expression is upregulated rendering VSMC more sensitive to gas6-induced proliferation and contributing to the formation of atherosclerotic plaques and arterial restenosis.

1.7.2.2 Role of gas6-Axl interaction in cell adhesion

In addition to its role in cell migration, gas6-Axl interactions can also promote cell adhesion to endothelium which could lead to development of pathological conditions such as rheumatoid arthritis (RA). Rheumatoid arthritis is characterized by hyperplasia of synovial cells and angiogenesis (Harris, 1990). Synovial inflammation in rheumatoid arthritis is characterized by angiogenesis and adhesion of inflammatory cells to endothelium (Koch, 1998). A study by O'Donnell et al. has demonstrated that gas6-Axl interactions play significant role in RA. The ability of gas6 to adhere to human umbilical vein endothelial cells and protect them from apoptosis sugests a functional role of gas6–Axl interactions in endothelial cells (O'Donnell et al., 1999). This study shows that synovial fluids of patients with rheumatoid arthritis have increased expression of Axl mRNA. The most striking expression of Axl was associated with vascular structures such as endothelial cells and smooth muscle cells. Rheumatoid arthritis patients also show

increased levels of gas6 in their synovial fluids. Thus, using endothelial cells as a model system, O'Donnell et al. was able to show that gas6 behaves as a survival factor in a cytotoxic environment i.e. in the presence of TNF-α. In a toxic environment such as the synovial fluid of rheumatoid arthritis patients, where there is a high concentration of inflammatory cytokines such as TNF-α, gas6-Axl interaction may have a protective effect of the vasculature. Gas6 promotes endothelial cell survival and contributes to the maintenance of pathological vasculature in rheumatoid arthritis.

A prior study by McCloskey et al. demonstrated that, in murine myeloid progenitor 32D cells, attachment of gas6 to the plasma membrane promotes Axl mediated cell adhesion to 32D myeloid cells expressing Axl and permitted cell aggregation in response to gas6. These effects were blocked by soluble Axl-ECD. (McCloskey et al., 1997). Aggregation was not observed in gas6 untreated cells devoid of the Axl receptor. Cell-to-cell binding was also blocked by the addition of calcium chelators, as expected for Gla-containing protein. In addition, using kinase deleted or inactive mutants of Axl, McCloskey et al have also demonstrated that the kinase domain of Axl is not required for the aggregation effect and that homotypic binding is solely due to the transmembrane and extracellular domains of Axl. The hypothesized mechanism of aggregation is gas6 binding to the surface of one cell via the Gla domain and simultaneously interacting with Axl on an adjacent cell. McCloskey et al. confirmed this hypothesis by showing that 32D cells expressing only gas6 but not Axl were able to form mixed cell aggregates with Axl expressing cells. Furthermore, using truncated mutants of gas6, they have shown that either the Gla and/or EGF domains of gas6 are required for adhesion of 32D Axl

expressing cells. Thus, this study has provided evidence that gas6 induces cell adhesion and that binding to the cell surface is Gla domain dependant.

Taken together, these two studies clearly demonstrate that gas6-Axl interaction may play a significant role in cell adhesion and that Gla domain of gas6 may contribute to this effect.

1.7.2.2.1 Role of gas6-Axl interactions in vascular adhesion

During vascular injury, macrophages and T lymphocytes adhere to the endothelium and undergo subsequent activation and chemotaxis through the endothelial cell layer. This activation in turn, induces migration and proliferation of VSMC leading to advanced lesions of atherosclerosis within the intima of the affected artery. The presence of gas6 in precursor T cell lines and monocytes suggests a potential role for activating endothelial cells during the initial inflammatory response following vascular injury (Dirks et al., 1999). On the contrary, high concentrations of gas6 inhibit granulocyte adhesion to endothelial cells, suggesting an anti-inflammatory effect of gas6–Axl interactions in endothelial cells (Avanzi et al., 1998).

Avanzi et al. performed a series of aggregation assays on Axl transfected M07e (a human leukemic factor-dependent cell line) cells in the presence and absence of rhgas6. M07e transfected Axl cells displayed homotypic aggregation, whereas wild-type M07e cells did not. Addition of rhgas6 to the aggregation assay completely inhibited cell aggregation. Moreover, to assess the rhgas6 effect on endothelial cell adhesion, Avanzi et al. performed adhesion assays in vitro between cultured endothelial cells and leukocytes. Results demonstrated that gas6 was able to significantly inhibit adhesion of leukocytes to

endothelial cells in the presence of chemoattractants such as phorbol 12-myristate 13-acetate (PMA), PAF, IL-1β, tumor necrosis factor-α (TNF-α), and thrombin. In contrast, gas6 was unable to promote the adhesion of leukocytes to endothelial cells in the presence of the chemoattractant, IL-8.The difference in adhesion activity of gas6 in the presence of various chemoattractants is due, in part, to the different mechanisms of action of adhesion of leukocytes to endothelial cells. One possibility suggests that gas6 activates Axl thereby inhibiting the endothelial response to proadhesive stimuli. Another possibility is that resting endothelial cells produce gas6 to play role in leukocyte migration control. When activated by pro-inflammatory stimuli, endothelial cells do not synthesize gas6 thereby promoting leukocyte binding and activation of proadhesive machinery. This mode of action was confirmed by an analysis showing elevated gas6 expression on the surface of resting endothelial cells and decreased expression on activated endothelium (Avanzi et al., 1998). In either case, gas6 may function as a physiologic anti-inflammatory agent.

1.7.3 Role of gas6-Axl in atherosclerosis

In addition to its role in rheumatoid arthritis, gas6-Axl interactions may have a significant function in the development of atherosclerosis. This interaction contributes to vascular pathophysiology by potentiating the proliferation of and preventing the death of VSMCs leading to the development of atherosclerotic lesions. A key event in the development of atherosclerosis is uptake of oxidized low density lipoprotein (OxLDL) by macrophages. Massive accumulation of lipid and subsequent foam cell formation is caused by modified low density lipoprotein (LDL) which acts as a ligand for scavenger

receptors located on macrophages and smooth muscle cells (Steinberg et al., 1989). Several macrophage scavenger receptors, such as CD36, CD68 and SRA (scavenger receptor A), are important in binding and internalizing OxLDL (Endemann et al., 1993; Ramprasad et al., 1995; Kodama et al., 1990).

During atherogenesis, VSMCs migrate from the media to the intima of the arterial wall, where they proliferate and accumulate lipid, becoming foam cells (Murao et al., 1999). As demonstrated by Murao et al. gas6 plays an important role in regulation of scavenger receptor expression and LDL uptake. This study showed that gas6 was able to increase the expression of scavenger receptor A (SRA) in human smooth muscle cells. This increase in SRA expression was correlated with receptor activity in these cells. Moreover, in the presence of OxLDL, gas6 expression was significantly elevated suggesting an important relationship between gas6-Axl and SRA.

In normal VSMCs, the expression of scavenger receptors is weak (Melagrano et al., 1998). However, in the presence of an appropriate stimulus, expression of the receptor is increased. For example, previous findings by Li et al. have demonstrated that, after balloon injury in rabbits, expression of scavenger receptors is only detected in the neointima of hypercholesterolemic rabbits clearly demonstrating that scavenger receptor expression is upregulated in smooth muscle cells in atherosclerotic lesions. Similarly, expression of Axl is increased in the neointima of VSMCs of rat carotid arteries after balloon injury (Melagrano et al., 1998).

Taken together, these findings suggest that gas6 can stimulate the expression of SRA in Axl-expressing human smooth muscle cells and affect formation of foam cells

and progression of atherosclerosis. However, the exact mechanism responsible for gas6-dependant SRA expression and foam cell formation has yet to be determined.

Initial evidence of the intracellular signalling pathways involved in gas6-Axl mediation of SRA expression was published by Ming et al. This study demonstrated that gas6 dependant expression of SRA is linked to Akt pathway activation. Moreover, it showed that gas6-induced expression of SRA was Axl dependant since the exposure of VSMCs to peptides that block the binding of gas6 to Axl completely abrogated gas6 induction of SRA.

Further support of Axl involvement was confirmed by studies using cells that do not express Axl. Exposure of these cells to gas6 failed to induce SRA expression unless they were trasfected with Axl. Furthermore, induction of SRA expression was PI3 kinase dependent since wortmannin, an inhibitor of PI3-kinase, abrogated gas6-induced SRA expression. The downstream target of PI3 kinase, namely Akt, was also affected; Dominant-negative Akt mutants blocked SRA expression whereas constitutively activated Akt induced SRA expression in the absence of gas6.

Thus, these results, taken together, clearly outline involvement of gas6-Axl in development of atherosclerotic lesions and suggest possible signalling pathways involved.

1.7.4 Role of gas6-Axl in protection from apoptosis

Gas6 and Axl are expressed in a variety of tissues and their interaction is important in many processes and disease states. In order to better understand the role of these molecules in different cell systems, it is necessary to dissect cell signaling

mechanisms triggered by their interaction. Once activated, gas6-Axl induces activation of several intracellular molecules responsible for cell survival and mitogenesis. Thus, one of the major roles of these molecules is in cell survival.

Initial studies of the role gas6-Axl in cell survival were conducted in NIH 3T3 fibroblasts by Goruppi et al. This group demonstrated that gas6 behaves not only as an anti-apoptotic factor but also as a mitogen. It induces Axl phosphorylation with subsequent activation of classical antiapoptotic pathways involving PI3 kinase and Akt (Grouppi et. al., 1996). Both, the mitogenic and antiapoptotic activities of gas6 correlates with its ability to interact with the NIH3T3 endogenous Axl receptor since both of these properties of gas6 can be abolished by a soluble Axl extracellular domain. A subsequent study by this group also showed that the mitogenic effect of gas6 is mediated thorough Ras, a protein important in cell proliferation and survival. This effect was inhibited by dominant negative Ras (Grouppi et al., 1999). This study also implicates involvement of GTPases, Rac and Rho, in gas6-mediated cell survival. The Rho GTPases form a subgroup of the Ras superfamily of 20- to 30-kD GTP-binding proteins that have been shown to regulate a wide spectrum of cellular functions (Van Aelst and D'Souza-Schorey, 1997). Activation of Rho GTPases is, in part, regulated by PI3 kinase (Hawkins et al., 1995). Grouppi et al. demonstrate that there is a strict requirement for the Rho family of GTPases in gas6-mediated survival of NIH 3T3 fibroblasts. In fact, they suggest that this activation is mediated by PI3 kianse since, both wortmannin and a p85 dominant negative construct efficiently block gas6 survival activities and mitogenic effects (Goruppi et al., 1997). Ras was indeed shown to be exclusively required for gas6-dependent mitogenesis (Grouppi et al., 1999). In addition, the study confirms initial findings that the anti-

apoptotic effect of gas6 is mediated by Akt and also shows activation of Bad, a pro-apoptotic Bcl-2 related protein. Bad has been shown to be a substrate of Akt in cytokine and growth factor induced survival (Datta et al., 1997). Goruppi et al. also show that gas6 is able to induce ERK activation during gas6-dependent survival and mitogenc activity. Similar mitogenic effect of gas6 have been found in Schwann cells where gas6 induces activation of ERK2, a part of raf/ras/MEK signalling pathway, to induce human Schwann cell growth as demonstrated by increasing cell number and thymidine incorporation (Li et al., 1996) confirming previous findings that gas6 acts as mitogenic factor.

Gas6 anti-apoptotic activity was previously shown to absolutely require the PI3 kinase and its substrate Akt in serum-starved NIH 3T3 mouse fibroblasts. Akt has been shown to drive transcriptional activation of nuclear factor kappa B (NF-κB). NF-κB is located in the cytoplasm of the majority of cell types as a homodimer or heterodimer of a family of structurally related proteins (Perkins, 2000). There are five members belonging to this family: RelA (p65), cRel, RelB, NF-κB1 (p50/p105), and NF-κB2 (p52/p100) all of which are present in an inactive form associated with inhibitory proteins (the IκB family) that mask their nuclear localization signal (Ozes et al., 1999). Upon growth factor stimulation, IκB is rapidly phosphorylated (Mercurio and Manning, 1999). This phosphorylation triggers their proteolytic degradation and releases the NF-κB dimer that can then translocate to the nucleus, where it directly binds to its DNA sequence to activate gene transcription. Deamrchi et al. demonstrate that gas6 can lead to NF-κB induction. They show that gas6 treatment is coupled to a decrease in IκB protein levels and an increase in NF-κB binding activity in NIH 3T3 fibroblasts. Furthermore, this

study demonstrates that NF-κB is required for gas6-mediated protection from apoptosis and that this activation involves PI3 kinase and Akt (Demarchi et al., 2001).

These initial findings give insight into the intricate nature of gas6-Axl signaling initiating interest in this area. Studies that followed confirmed the survival effect of gas6-Axl interactions in several different cell systems. Bellosta et al. confirmed that gas6 acts as a survival factor in serum-depleted NIH 3T3 fibroblasts. If constitutively expressed, gas6 does not have the ability to induce cell transformation or uncontrolled growth. Moreover, once phosphorylated in response to gas6, Axl was able to induce MAP kinase activation which resulted in an increased survival affect of gas6 (Bellosta et. al., 1997). Gas6 activity strictly correlates with its ability to activate Axl since fibroblasts from Axl-null mice resulted in higher serum-deprivation induced apoptosis.

Ark, a mouse homologue of Axl, has an anti-apoptotic role in neurons that release gonadotropin-releasing hormone (GnRH) in response to gas6 (Allen et al., 1999). This study shows that Ark-negative cells are more susceptible to serum-depletion induced apoptosis than Ark-positive cells in response to gas6. Furthermore, once activated by gas6, Ark was able to induce activation of ERK (extracellular signal-regulated kinase) and Akt, signaling molecules responsible for cell survival. In a follow up study, Allen et al. demonstrated that gas6/Ark signalling promoted activation of the Rho family GTPase Rac, as previously observed in NIH 3T3 fibroblasts. Also, p38 MAPK, implicated in neuronal apoptosis, was activated downstream of Ark and Rac (Allen et al., 2002).

In addition to its antiapoptotic role in NIH 3T3 fibroblasts, Schwan cells and GnRH neurons, gas6 also plays important role in the vascular system. O'Donell et al. has

demonstrated that gas6-Axl interactions play a significant role in rheumatoid arthritis. Patient suffering from this condition showed increased expression of both Axl and gas6 in their synovial fluids. Moreover, this study confirmed the anti-apoptoic activity of gas6 in the vascular system by showing that gas6 was able to rescue human umbilical vein endothelial cells (HUVECs) after complete serum depletion for up to five days (O'Donell et al., 1999). Also, gas6 was able to rescue HUVECs from tumor necrosis factor α (TNF-α) mediated apoptosis, a molecule known to induce apoptosis in some cell types. Thus, this study was the first to show that gas6 acts as a survival factor on endothelium but offered no insight into the mechanistic nature of this effect. Similar results were confirmed in pulmonary artery endothelial cells where gas6 induced both proliferation and survival (Healy et al., 2001). A study of gas6-Axl interactions in vascular smooth muscle cells confirmed previous findings in NIH 3T3 fibroblasts that gas6 protection is mediated through the activation of PI3 kinase and Akt but found no significant role of ERK in this antiapoptotic effect (Melaragno et al., 2004).

Taken together, these studies demonstrate that gas6-Axl interaction have similar function, antiapoptotic and mitogenic, in several different cell systems. This interaction triggers the classical antiapoptotic pathway including activation of PI3 kinase, Akt, NFκB and the Bcl-2 family of proteins. In addition to its antiapoptotic function, gas6 may act as a mitogenic factor in some cell systems by activating MAP Kinase and the ERK pathway. As seen in this review, gas6-Axl interactions have a myriad of functions in several different systems but the main goal of this work is to examine the anti-apoptotic function of gas6 in endothelial cells. Moreover, the following two chapters will provide more clues into the antiapoptotic mechanism of gas6-Axl interactions during serum

depletion and for the first time show how the unique posttranslational modification of gas6, namely, γ-carboxylation, may alter the survival property of gas6 in endothelial cells.

Chapter 2

Intracellular signaling pathways involved in gas6-Axl-mediated survival of endothelial cells

ABSTRACT

Gas6 is a γ-carboxylated ligand for the receptor tyrosine kinase Axl. Gas6-Axl interactions can rescue endothelial cells from apoptosis, and this study examined the intracellular signalling mechanisms responsible for this phenomenon. Using flow cytometry, we first confirmed that gas6 can abrogate apoptosis induced by serum starvation of primary cultures of human umbilical vein endothelial cells (HUVECs). This effect is mediated through phosphorylation of the serine-threonine kinase Akt, with maximal phosphorylation observed after 4 h of treatment with 100 ng/ml gas6. Inhibition of Akt phosphorylation and abrogation of gas6-mediated survival of HUVECs by wortmannin implicated phosphatidylinositol 3-kinase as the mediator of Akt phosphorylation. Dominant negative Akt constructs largely abrogated the protective effect of Gas6 on HUVECs, underscoring the importance of Akt activation in gas6-mediated survival. Several downstream regulators of this survival pathway were identified in HUVECs, namely, NF-κB as well as the antiapoptotic and proapoptotic proteins Bcl-2 and caspase 3, respectively. We showed that NF-κB is phosphorylated early after gas6 treatment as evidenced by doublet formation on Western blotting. As well, the level of Bcl-2 protein increased, supporting the notion that the Bcl-2

antiapoptotic pathway is stimulated. The levels of expression of the caspase 3 activation products p12 and p20 decreased with gas6 treatment, consistent with a reduction in proapoptotic caspase 3 activation. Taken together, these experiments provide new information about the mechanism underlying gas6 protection from apoptosis in primary endothelial cell cultures.

INTRODUCTION

Gas6 is a ligand for the receptor tyrosine kinase Axl (41). It contains four protein domains that comprise, from the NH_2 to COOH terminus, a γ-carboxyglutamic acid-containing domain, two epidermal growth factor-like domains, and a COOH-terminal steroid hormone binding globulin-like domain. This structure is homologous to that of protein S, a vitamin K-dependent blood coagulation protein and, like the vitamin K-dependent proteins, contains several γ-carboxyglutamic acid residues at its NH_2 terminus.

Gas6 is a ligand for the receptor Axl (18, 40), which is a member of a family of receptor tyrosine kinases that include c-mer and rse. Axl was first isolated from a T-cell leukemia cell line in 1991 (35) and is a type I transmembrane protein whose extracellular portion is composed of two immunoglobulin-like domains and two fibronectin type III domains (18, 40). Gas6-Axl interactions have been examined in a variety of different cell systems. The pleotropic effects of gas6-Axl interactions in different tissues may be summarized as follows. In vascular smooth muscle, gas6 was initially purified as a mediator of vascular smooth muscle proliferation (34). Later studies showed Axl upregulation at sites of vascular injury, suggesting a role for this receptor in vascular remodeling (29). In the kidney, gas6-Axl interactions are important for mesangial cell proliferation (43) and play a role in nephrotoxic glomerular injury (45) as well as diabetic nephropathy (32). Interestingly, one intracellular signaling pathway that mediates mesangial cell proliferation occurs through signal transducer and activator of tranduction 3 (STAT3) activation (44). Other tissues where gas6 may play a functional role include bone, where gas6-Axl interactions upregulate osteoclast function (27, 33), the central

nervous system, where gas6 protects neurons from amyloid-induced apoptosis (42), and the eye, where gas6 mediates outer retinal pigment epithelial function (22, 23). Finally, homozygous null gas6 mice have a platelet dysfunction that protects mice against lethal intravascular thrombosis (1).

The role of gas6-Axl interactions in endothelial physiology is discussed below. The sequence and nature of the mitogenic and antiapoptotic events resulting from gas6-Axl interactions have been most extensively studied in murine NIH-3T3 fibroblasts (19–21). In this system, gas6-Axl interactions activate Akt through phosphorylation. Akt itself is a serine-threonine kinase that has been shown to be a key intracellular regulator of cellular survival. Its activation by phosphorylation is carried out by phosphatidylinositol 3-kinase (PI3K), a kinase that can be activated by upstream events such as ligand-receptor interactions or through the recruitment of adaptor proteins to activated cell surface receptors. Activation of Akt leads to downstream signaling events, including those associated with the mitochondrial regulation of apoptosis (7).

Recent studies of endothelial cell physiology have demonstrated that the endothelium undergoes apoptosis. Information about mechanisms underlying the regulation of endothelial cell survival is fundamentally important for understanding angiogenesis and vascular remodeling, crucial processes in a wide variety of disease states ranging from atherosclerosis to tumor metastasis. Known inducers of endothelial apoptosis include TNF-α, oxidized LDL, and reactive oxygen species. On the other hand, VEGF, angiopoietin 1, basic FGF, and insulin have all been shown to have antiapoptotic/survival effects on endothelium (4). Both VEGF and angiopoietin-mediated protection from apoptosis in endothelial cells have been particularly well studied (15–17,

38). These mediators utilize classical survival pathways such as Akt phosphorylation and NF-κB activation, suggesting that these pathways are functional in endothelium (16, 38).

The present study concerns the nature of intracellular signaling pathways responsible for gas6-Axl-mediated protection from apoptosis. Although mouse NIH-3T3 fibroblasts have been used as a model system for studying the antiapoptotic effects of gas6-Axl interactions, the present experiments address cell signaling events using freshly isolated primary human endothelial cells maintained for limited periods in culture. The results underscore the important antiapoptotic role of gas6 and demonstrate that this antiapoptotic effect involves "classical" survival pathways including Akt phosphorylation, NF-κB activation, Bcl-2 stimulation, and, ultimately, caspase 3 inhibition.

MATERIALS AND METHODS

Cells

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords by collagenase digestion (26) and cultured in complete medium comprising endothelial cell basal medium (EBM-2) supplemented with an endothelial cell Bullet Kit (Cambrex) containing 2% FCS, human endothelial growth factor-2 (EGF-2), human fibroblast growth factor-2, human vascular endothelial growth factor, R3-insulin-like growth factor I, ascorbic acid, hydrocortisone, heparin, gentamicin, and amphotericin B [endothelial cell growth medium (EGM-2)]. Cultures were passaged in Corning tissue culture dishes coated with 0.1% gelatin (Sigma). Cells were grown at 37°C in a humidified atmosphere with 5% CO_2. HUVECs used for the experiments described herein were passaged between three and six times. For serum starvation, EGM-2 was replaced by EBM-2 without supplements.

Detection and quantification of apoptosis

For analysis of apoptosis by flow cytometry, 1 x 10^6 cells were plated in 100-mm tissue culture plates. Upon reaching 70% confluency, cells were placed in serum-free media in the presence or absence of 100 ng/ml recombinant human gas6 and incubated for 24, 48, and 72 h. The cells were then harvested by trypsinization and washed twice with PBS (GIBCO Invitrogen). Apoptosis was quantified by flow cytometry either by costaining harvested HUVECs with FITC-conjugated annexin V and propidium iodide (BD Biosciences) or by staining with propidium iodide only and enumerating the hypodiploid apoptotic cells in the sub-G_1 fraction. Cells analyzed by flow cytometry

(Becton Dickinson) were quantified using Cell Quest software. For experiments performed with wortmannin, 1 μM wortmannin (Sigma) was present in the medium for 30 min before gas6 treatment and/or serum starvation. For experiments performed with dominant negative Akt constructs, HUVECs were transfected with adenoviral constructs containing dominant negative Akt that were obtained as a gift from Dr. S. Richard at the Lady Davis Institute for Medical Research.

Western blot analysis

HUVECs were grown in 100-mm dishes. After reaching 70% confluency (5×10^6 cells), they were serum starved for 0.5, 1, 4, 24, and 48 h in the presence or absence of 100 ng/ml recombinant human gas6. HUVECs were trypsinized, pelleted at 300 *g*, and then lysed by the addition of 150 μl lysis buffer [50 mM NaF, 50 mM Tris·HCl (pH 7.5), 1% Igepal, 0.1 mM EDTA (pH 8.0), 150 mM NaCl, 10 mM $NaPO_4$, 10% glycerol, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride plus Complete Protease Inhibitor Cocktail (Roche)]. Samples comprising equal amounts of total protein were analyzed by 7.5% and/or 12% SDS-PAGE and then transferred to a nitrocellulose membrane (Bio-Rad). Western blot analysis was carried out using the following polyclonal antibodies and dilutions: anti-NF-κB p65 (1:500), anti-Bcl-2 (1:1,000), and anti-caspase 3 (1:1,000) (Santa Cruz Biotechnology) as well as anti-Akt (1:1,000) and anti-phospho-Akt (1:1,000) (Cell Signaling Technology) rabbit polyclonal antibodies followed by the addition of a goat anti-rabbit peroxidase conjugated secondary antibody (Santa Cruz Biotechnolgy). The blots were developed with an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

RESULTS

The present work examines the intracellular signaling pathways in endothelial cells that are important for Gas6-Axl-mediated protection from apoptosis. Axl is present in freshly isolated HUVECs (cells passaged 3–6 times) as confirmed by Western blot analysis (data not shown). These results confirm previous findings that demonstrate Axl expression by Western blot analysis in HUVECs (36) and by Northern blot analysis as well as Western blot analysis in human pulmonary endothelial cells (24).

Effect of Gas6 on apoptosis induced by serum starvation

When HUVECs were cultured in serum-free medium for 72 h, 55% of cells underwent apoptosis as detected by annexin V staining. This is apparent from the comparison of the flow cytometric analysis shown in Fig. 1*A* (complete medium) to that in Fig. 1*B* (serum-free medium). However, if gas6 (100 ng/ml) was included in the serum-free medium, only 11.5% of cells underwent apoptosis (Fig. 1*C*). The time course of apoptosis is shown in Fig. 1*D* (mean of 4 experiments). These results indicate that serum starvation induces apoptosis in primary HUVECs in culture and that this can be abrogated with the addition of gas6.

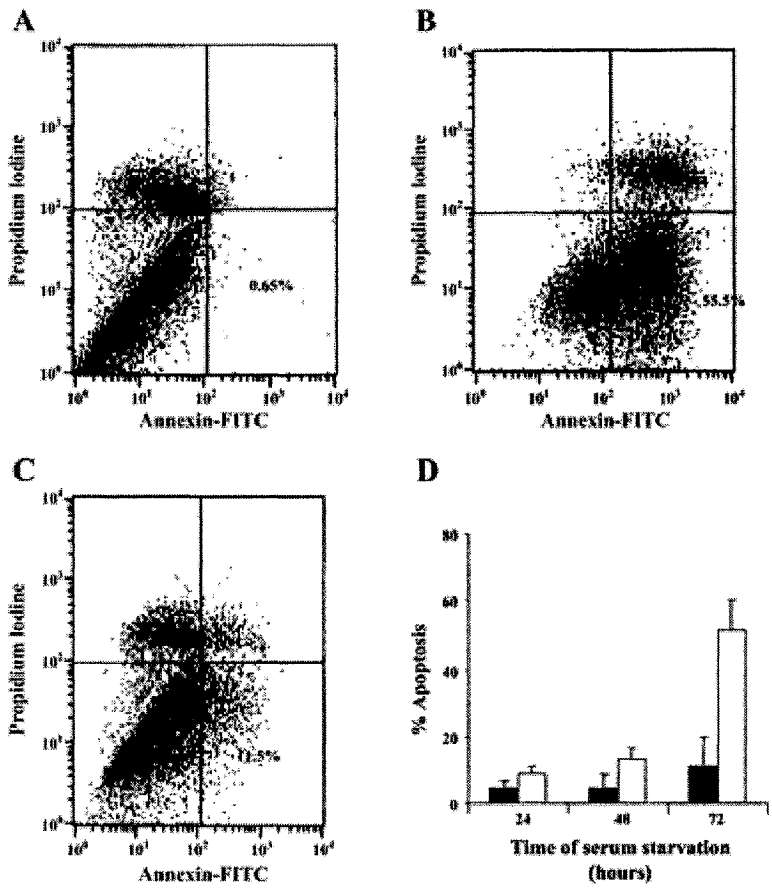


Figure. 2-1. Gas6 is a survival factor for human umbilical vein endothelial cells (HUVECs). HUVECs were grown in culture as described in MATERIALS AND METHODS. To measure apoptosis, the cells were stained with FITC-conjugated annexin V and propidium iodide, and the percentage of apoptotic cells (annexin V positive; propidium iodide negative) were determined on a Becton Dickinson FACS Analyzer. *A*: HUVECs in 2% FCS containing supplemental growth factor medium as outlined in MATERIALS AND METHODS. *B*: HUVECs were serum starved without supplemental growth medium for 72 h before being analyzed by flow cytometry. *C*: serum-starved HUVECs as described in *B* but with the addition of 100 ng/ml recombinant human Gas6. The percentage of apoptotic cells is indicated in the *bottom right* quadrant. The results shown are representative of 4 independent experiments. *D*: HUVECs were treated for 24, 48, and 72 h in serum-free media (open bars) or serum-free media supplemented with 100 ng/ml recombinant human Gas6 (solid bars). The percentage of apoptotic cells was then analyzed by flow cytometry as described above. Data shown are means ± SE of 4 independent experiments.

Role of Akt in Gas6-mediated protection from apoptosis

We then examined the intracellular signaling pathways responsible for this gas6-mediated protection from apoptosis. Because VEGF and angiopoietin mediate protection of endothelial cells via survival pathways involving Akt activation, (16, 38), the participation of Akt in the signaling pathways activated by Gas6 was examined. Akt is activated through phosphorylation at Ser^{473} and Thr^{308}. As shown in Fig. 2*A*, Akt remained phosphorylated as cells survived in culture but became dephosphorylated as cells underwent apoptosis under serum deprivation. However, in the presence of 100 ng/ml gas6, Akt remained phosphorylated for up to 72 h, as depicted in Fig. 2*B*. Gas6 phosphorylation of Akt was dose dependent, with maximal phosphorylation at 100–200 ng/ml Gas6 (Fig. 3). Furthermore, the experiment shown in Fig. 4 indicates that a dominant negative Akt construct resulted in a marked reduction in the protective effect of gas6 on apoptosis in HUVECs, thus providing clear evidence for a critical role of the PI3K/Akt pathway for gas6-mediated survival in HUVECs.

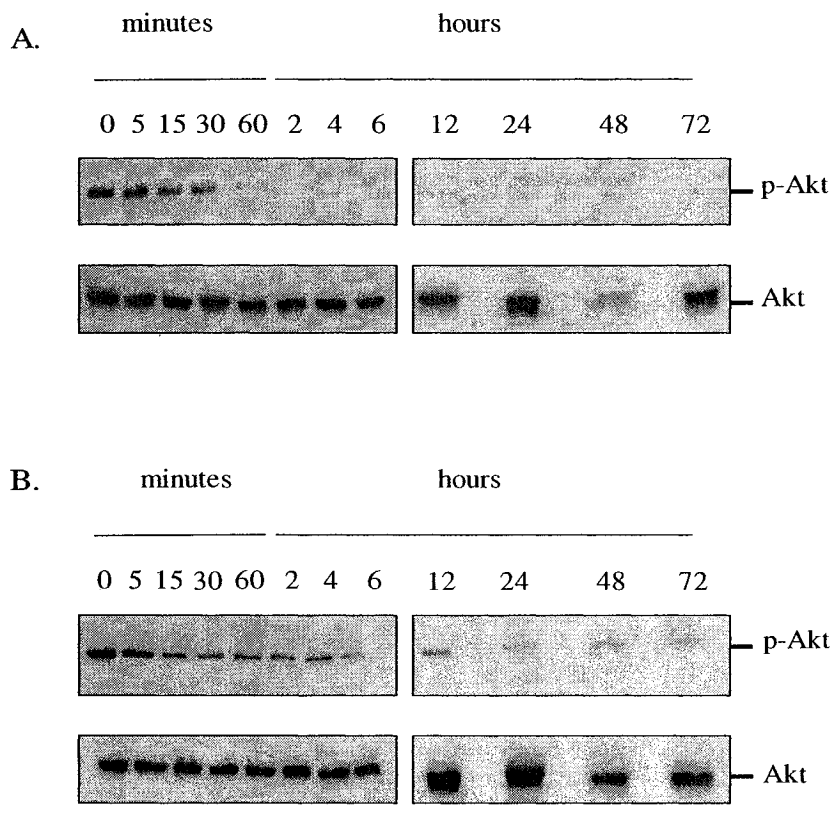


Figure. 2-2. Gas6 phosphorylates Akt in HUVECs during serum starvation. HUVECs were serum starved for the indicated time points in the absence (*A*) or presence (*B*) of 100 ng/ml Gas6, after which the whole cell lysates were subjected to SDS-PAGE and immunoblotted with antibodies against Akt and phospho-Akt (p-Akt) as described in MATERIALS AND METHODS. The experiment shown is representative of 1 of 3 performed.

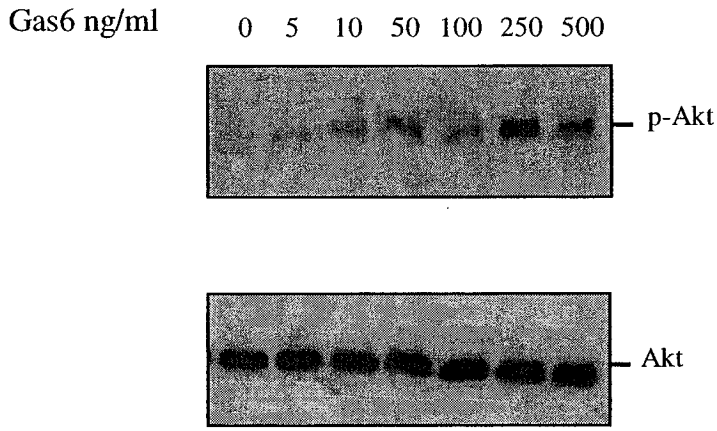


Figure 2-3. Concentration dependence of Gas6-mediated phosphorylation of Akt. HUVECs were serum starved for 4 h in the presence of varying concentrations of Gas6 as indicated. Their lysates were subject to SDS-PAGE followed by Western blotting as described in Fig. 2. The experiment shown is representative of 1 of 3 performed.

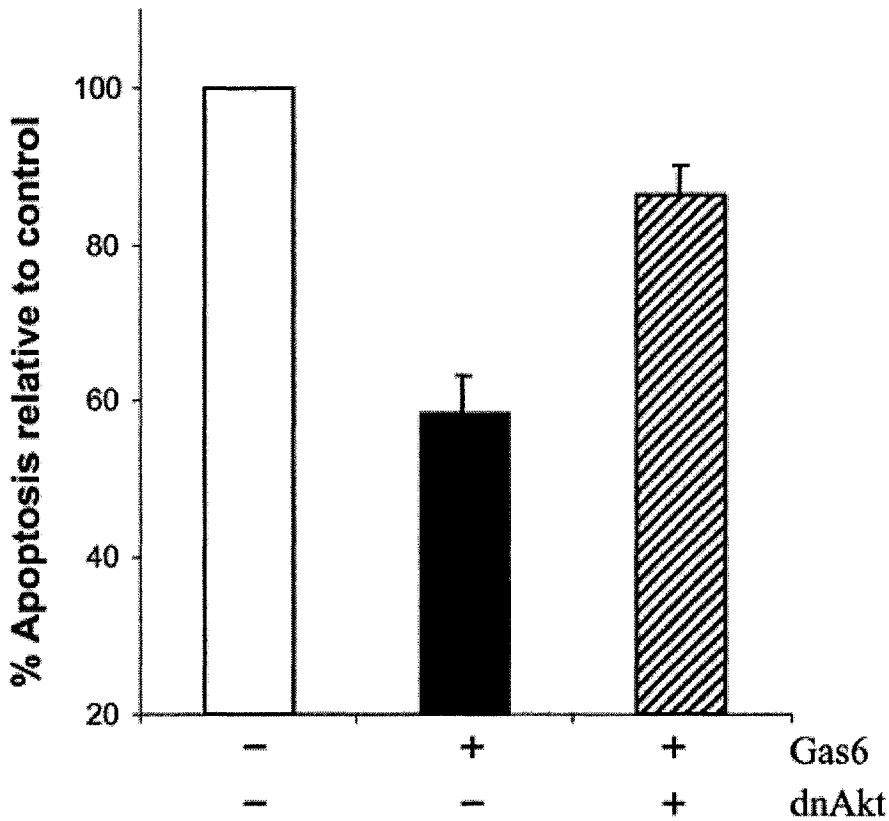


Figure 2-4. Akt mediates the survival effect of Gas6 on HUVECs. HUVECs were serum starved for 48 h in the presence of 100 ng/ml Gas6 and/or transfected with dominant negative Akt (dnAkt). The percentage of cells undergoing apoptosis by sub-G_1 analysis was measured by flow cytometry as outlined in MATERIALS AND METHODS. Data shown are means ± SE of 3 independent experiments each normalized to the controls without Gas6.

Akt has been shown to be phosphorylated by PI3K (3, 13). To demonstrate whether PI3K phosphorylates and activates Akt in HUVECs, we examined the effect of wortmannin, a specific PI3K inhibitor, on Akt phosphorylation. Figure 5 shows the time course of Akt phosphorylation in the presence and absence of 100 ng/ml Gas6 with (*B*) and without wortmannin (*A*). As shown, 1 μM wortmannin largely abrogated Akt phosphorylation induced by Gas6, indicating that Gas6 activates Akt through PI3K. To confirm that the survival effect of Gas6 is dependent on PI3K, apoptosis was measured in the presence of Gas6 alone and in the presence of Gas6 plus wortmannin. As shown in Fig. 5*C*, a significant Gas6-mediated protection of HUVECs from serum starvation-induced apoptosis was not detected in the presence of 1 μM wortmannin.

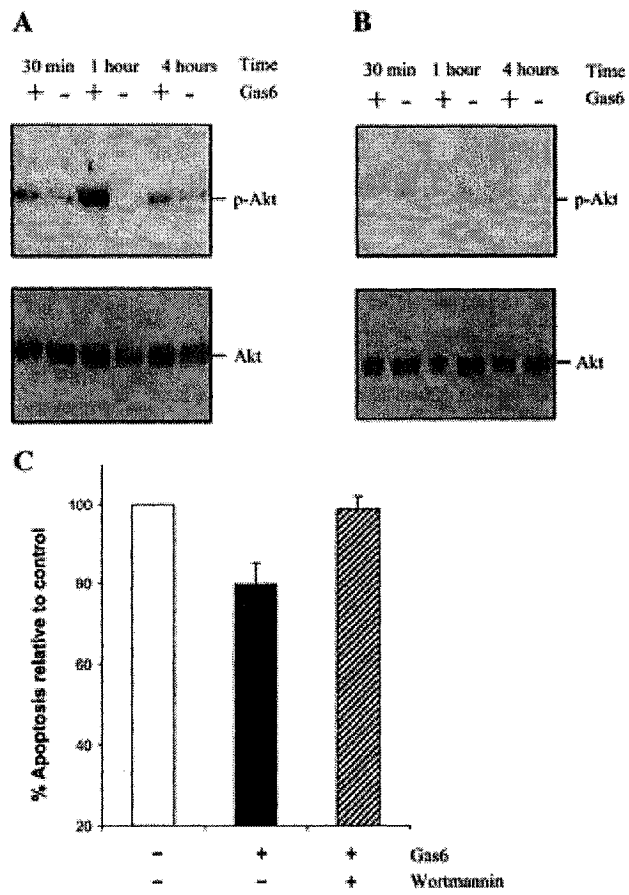


Figure 2-5. Gas6 stimulates Akt phosphorylation through phosphatidylinositol 3-kinase (PI3K). *A*: HUVECs were serum starved for the indicated times in the absence and presence of 100 ng/ml Gas6. Cell lysates were then subjected to SDS-PAGE followed by Western blotting as described in Fig. 2. *B*: HUVECs were incubated as in *A* but pretreated with wortmannin as described in MATERIALS AND METHODS. The experiment shown is representative of 1 of 3 performed. *C*: HUVECs were serum starved in the absence (control) or presence of 100 ng/ml Gas6 with or without 1 μM wortmannin for 72 h, and the percentage of cells undergoing apoptosis by sub-G_1 analysis was measured by flow cytometry as outlined in MATERIALS AND METHODS. Data shown are means ± SE of 3 independent experiments each normalized to the controls without Gas6.

Downstream mediators of Gas6-Axl-mediated survival in endothelial cells

Downstream mediators of cell survival in HUVECs cultured in the presence of Gas6 were then analyzed. The experiments shown in Fig. 6 were designed to examine the participation of NF-κB, a well-known effector of cell survival that is active when phosphorylated. As shown in Fig. 6*A*, Gas6 addition to serum-starved cells resulted in the phosphorylation of the p65 subunit of NF-κB. Phosphorylation is evident by the doublet seen with Gas6 treatment. In the absence of Gas6, NF-κB remained a singlet (Fig. 6*B*). This phosphorylation of NF-κB occurs early and is maintained for up to 48 h. This finding provides evidence for the involvement of NF-κB in Gas6-mediated endothelial cell survival. To establish more downstream effectors of cell survival induced by Gas6-Axl interactions, two well-described proteins important in apoptosis were examined, namely, Bcl-2 and caspase 3. Bcl-2 is an antiapoptotic protein whose mechanism of action is to prevent cytochrome *c* release from mitochondria, thereby preventing apoptosis. As shown in Fig. 7*A*, Bcl-2 protein expression was reduced during apoptosis induced by serum starvation, and, after treatment with Gas6, Bcl-2 protein expression was increased.

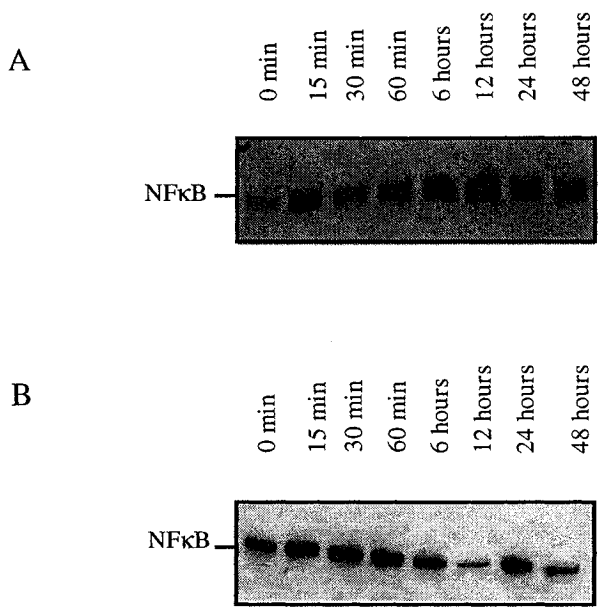


Figure 2-6. Gas6 activates NF-κB during endothelial cell survival. HUVECs were serum starved for the indicated time points in the presence (*A*) and absence (*B*) of 100 ng/ml Gas6. Lysates were then subjected to SDS-PAGE, followed by Western blotting with antibodies to the p65 subunit of NF-κB as described in Materials and Methods. The experiment shown is representative of 1 of 3 performed.

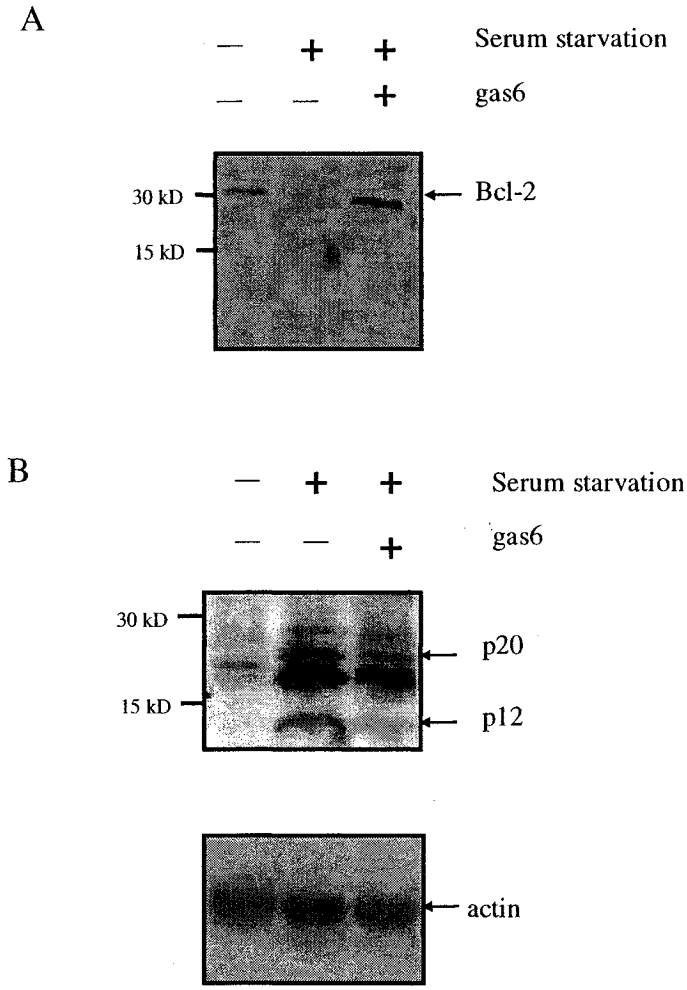


Figure 2-7. Gas6 increases Bcl-2 protein levels and reduces caspase 3 activation during protection of endothelial cells from apoptosis. HUVECs were serum starved for 24 h in the presence and absence of 100 ng/ml recombinant human gas6. Lysates were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with antibodies to Bcl-2 (*A*) or caspase 3 (*B*) as described in MATERIALS AND METHODS. Also shown are lysates from nonapoptotic, non-serum-starved HUVECs as well as protein loading controls detected with anti-actin antibodies (Santa Cruz Biotechnology). The experiment shown is representative of 1 of 3 performed.

In contrast to these findings, caspase 3 activation, a known effector of cell death, was reduced by gas6 treatment (Fig. 7*B*). Pro-caspase 3, the zymogen, is activated to caspase 3, which consists of two subunits, p12 and p20. Both of these activation products are increased during apoptosis (2). However, in the presence of 100 ng/ml gas6, these activation products were significantly reduced. The decrease in levels of p12 and p20 indicates a reduction in caspase 3 activation consistent with gas6's antiapoptotic effect.

DISCUSSION

Gas6 is a novel vitamin K-dependent protein that is a ligand for the receptor tyrosine kinase Axl (18, 40, 41). One property of gas6 is to mediate endothelial cell survival induced by serum starvation or TNF-α treatment (24, 36). To further our understanding of the role of endothelial cell survival in the etiology of atherosclerosis and in vascular development (4, 14), the experiments described in this paper were aimed to address the nature of the endothelial cell intracellular signaling pathways that are activated by gas6-Axl interactions.

Intracellular signaling pathways mediated by gas6-Axl interactions have previously been studied in NIH-3T3 fibroblasts (19–21), immortalized vascular smooth muscle cells (31), and mesangial cells (44). The first two cell systems are immortalized cell lines that are unlikely to resemble closely their primary cell counterparts. Mesangial cells, albeit primary cells, are derived from the murine kidney and involve the STAT3 activation pathway (44). The cells used in the present study are primary cells derived from freshly isolated human endothelium and were used within a very short period thereafter, i.e., three to six passages. Accordingly, these cells (HUVECs) represent a cellular phenotype that is more physiologically relevant. In the present study in which apoptosis was induced by serum starvation, we have shown both endothelial cell apoptosis induced by serum starvation and the rescue from apoptosis imparted by gas6-Axl interactions. The latter is based on the following observations: *1*) exogenously added gas6 abrogated endothelial cell apoptosis; *2*) endogenous Axl is expressed in HUVECs;

and *3*) gas6-mediated survival of endothelial cells has been shown to be mediated through Axl (24, 36).

The present study shows that gas6 rescues HUVECs from apoptosis through PI3K activation and subsequent Akt phosphorylation. Involvement of Akt in the signaling pathways of other mediators of endothelial cell survival has been reported. Thus VEGF protects endothelial cells from apoptosis via Akt/PI3K intracellular signaling events (16). The same holds true of insulin, another mediator of endothelial cell survival that prevents TNF-α-induced apoptosis (25). In the present study, wortmannin inhibited Akt phosphorylation and subsequent Gas6-mediated protection of HUVECs from apoptosis (see Figure 5), underscoring an important role of PI3K in Akt phosphorylation and Gas6-mediated HUVEC survival (3, 13). This is consistent with other studies examining Akt phosphorylation in endothelial cells. For example, wortmannin largely abrogated Akt phosphorylation in endothelial cells induced by shear stress (10). Furthermore, experiments with dominant negative Akt constructs (see Figure 4) demonstrate that Akt activation is necessary for mediating the survival effect of gas6.

Our experiments have identified several downstream signaling proteins involved in gas6-mediated cell survival, presumably via Akt phosphorylation. Thus, in HUVECs, gas6-mediated protection from apoptosis resulted in increased levels of Bcl-2 protein expression. The increase in Bcl-2 protein is similar to the protection from apoptosis mediated by VEGF (16). Another potential downstream target of Akt is NF-κB (37, 39). NF-κB is known as a ubiquitous regulator of gene expression in both inflammation and cell survival in many different cellular systems (30). It has also been shown to be active in vascular biology (8). For example, NF-κB activation has been linked to antiapoptosis

in endothelial cells, although some mediators, such as TNF-α, activate antiapoptotic pathways in endothelial cells via Akt that are independent of NF-κB (28). In the present study, NF-κB phosphorylation occurs early in gas6-mediated rescue of HUVECs from apoptosis, consistent with the rapid upregulation of NF-κB seen in NIH-3T3 fibroblasts (9).

During apoptosis, one of the final biochemical events leading to programmed cell death is the activation of the caspase cascade and generation of caspases from zymogens or procaspases. The resultant caspases are cysteine proteases whose peptide bond cleavage site precedes aspartic acid residues. These proteins are responsible for affecting such biochemical processes as DNA fragmentation, nuclear membrane breakdown, and mitochondrial damage (2). Caspase 3 is one of the final caspases that is activated leading to apoptosis. As shown in Figure 7*B*, gas6 treatment of HUVECs undergoing apoptosis resulted in a reduction in the formation of its active p12 and p20 components, consistent with a reduction in caspase 3 activation. The results are consistent with gas6's antiapoptotic effect and are consistent with a key role of this protease described in several other cell systems. For example, shear stress promotes endothelial cell survival and does so by reducing caspase 3 activity (11).

The importance of gas6-Axl interactions in endothelial cell physiology is evidenced in Gas6-mediated protection of HUVECs and human pulmonary artery endothelial cells from both serum starvation and TNF-α-induced apoptosis (24, 36). The question of whether gas6-Axl is involved in the protection of endothelial cells from other apoptotic stimuli that are more physiologically relevant is a timely issue. It is notable that protection from serum-starved apoptosis in endothelial cells is effected by acidification

(5), and D'Arcangelo et al. (12) have shown that this protection is associated with gas6-Axl interactions. Studies in our laboratory (preliminary experiments, not shown) suggest that Gas6 protects HUVECs from reactive oxygen species-induced apoptosis. Ongoing studies are underway to determine whether the signaling pathways described in the present study underlie gas6-mediated protection of HUVECs from apoptotic stimuli other than serum starvation.

In conclusion, this study shows that gas6-Axl interactions promote endothelial cell survival through Akt phosphorylation, NF-κB activation, increased Bcl-2 protein expression, and a reduction in caspase 3 activation. A most intriguing feature of gas6 as a mediator of cell survival is its unusual posttranslational modification, γ-carboxylation. The question of the role this modification plays in gas6 modulation of cell survival, particularly in endothelial cells, is currently under investigation.

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PREFACE TO CHAPTER 3

Although we had characterized the signaling pathway involved in gas6-Axl protection of endothelial cell apoptosis during serum stress our results did not examine the importance of γ-carboxylation of gas6 in this protective function. γ-carboxylation is a unique posttranslational modification of gas6 in which a glutamic acid residue is post-translationally modified by the vitamin K-dependent carboxylase. All the vitamin K-dependent γ-carboxylated (Gla) proteins contain 9–12 Gla residues that reside in the N-terminal and allow Gla containing proteins to bind to phospholipid membranes (Furie and Furie, 1998). Upon binding calcium, the Gla domain undergoes a change in tertiary structure and a series of hydrophobic residues at the N-terminus become exposed to solvent. These residues constitute, in part, a phospholipid-binding site (Freedman et al, 1996).

While the role of the Gla domain in soluble blood coagulation proteins has been clarified, its role in the function of gas6 is largely unknown. Chapter 3 concerns itself with the importance of Gla domain in the anti-apoptotic function of gas6.

Chapter 3

The role of γ-carboxylation in the anti-apoptotic effect of gas6 in endothelium

ABSTRACT

Gas6 is a novel member of the vitamin K-dependent family of γ-carboxylated proteins and is a ligand for the receptor tyrosine kinase Axl. Gas6–Axl interactions have been shown to mediate cell survival in vascular endothelium. Although the receptor-binding portion of gas6 lies in the C-terminus, the significance of the N-terminal γ-carboxylated residues (Gla domain) is not clear. To address this question, this study examines the role of the Gla domain in phospholipid binding as well as in the promotion of cell survival, especially in endothelial cells. The results show that carboxylated gas6 binds to phosphatidylserine-containing phospholipid membranes in an analogous manner to other γ-carboxylated proteins whereas decarboxylated gas6 does not. The γ-carboxylation inhibitor warfarin abrogates gas6-mediated protection of NIH3T3 fibroblasts from serum starvation-induced apoptosis. Furthermore, the role of γ-carboxylation in gas6's survival effect on endothelium is demonstrated directly in that only carboxylated, but not decarboxylated, gas6 protects endothelial cells from serum starvation-induced apoptosis. γ-carboxylation is also required for both Axl phosphorylation and PI_3 kinase activation. Taken together, these findings demonstrate that γ-carboxylation is necessary not only for gas6 binding to phospholipid membranes, but also for gas6-mediated endothelial cell survival.

INTRODUCTION

The growth arrest-specific 6 gene product (gas6) is a novel vitamin K-dependent protein [1]. It is an extracellular ligand for a family of receptor tyrosine kinases that include Axl (Ark, Ufo, Tyro7), Rse (Tyro3, Dtk, Etk, Brt, Tif), and Mer (C-mer, Nyk, Eyk) [2,3]. Gas6 is comprised of several protein domains which include, from N- to C-terminus, a γ-carboxyglutamic acid containing domain, four epidermal growth factor-like domains and a C-terminal steroid hormone-binding globulin-like domain. This structure is homologous to that of protein S, a vitamin K-dependent blood coagulation protein, and, like the vitamin K-dependent proteins, contains several γ-carboxyglutamic acid (Gla) residues at its N-terminus [1].

γ-carboxyglutamic acid is a glutamic acid residue that is post-translationally modified by vitamin K-dependent γ-carboxylation. γ-carboxylated proteins are primarily involved in blood coagulation and the importance of γ-carboxylation is highlighted by the clinical use of warfarin, an inhibitor of γ-carboxylation and effective oral anticoagulant [4]. All the vitamin K-dependent γ-carboxylated blood coagulation proteins contain 9–12 Gla residues that reside in the N-terminal 40–50 amino acids and allow Gla containing proteins to bind to phospholipid membranes [5,6]. Upon binding calcium, the Gla domain undergoes a change in tertiary structure and a series of hydrophobic residues at the N-terminus become exposed to solvent. These residues constitute, in part, a phospholipid-binding site [7]. More recent reports suggest that Gla domains also mediate protein–protein interactions in enzymatic complexes in blood coagulation [8–10].

Axl was first isolated from a T-cell leukemia cell line in 1991 [11] and is a type I transmembrane receptor whose extracellular portion is composed of both two immunoglobulin-like domains and two fibronectin type III domains, and whose intracellular short cytoplasmic tail has tyrosine kinase activity. Axl has a role in reproductive development as evidenced by recent murine knockout data where all three receptors (i.e. Axl, Rse, and Mer) are deleted [12]. Another phenotype has also been described, one in which these same mice demonstrate immune dysregulation characterized by autoimmunity and a lymphoproliferative disorder [13].

Gas6–Axl interactions have protean effects in many tissues including platelets [14], kidney [15–17], neurons [18], bone [19], vascular smooth muscle [20–24], the eye [25,26], and endothelium [27–30]. Gas6-deficient mice [14], similar to mice deficient in one of its receptors, Mer [31], demonstrate a platelet-signaling defect that protects these mice from lethal venous thromboembolism. The importance of gas6–Axl interactions in endothelial cell physiology is beginning to be understood. Gas6 mRNA is expressed in human endothelium [1]. We have recently shown that gas6–Axl interactions protect human umbilical vein endothelial cells (HUVECs) in culture from serum starvation-induced apoptosis utilizing classical intracellular signaling pathways [28–30]. Other data show that acidification-induced protection of endothelial cells from apoptosis is mediated by gas6–Axl [27]. Furthermore, Axl is upregulated at sites of vascular injury after angioplasty [32] and gas6–Axl interactions mediate hydrogen peroxide-induced apoptosis of endothelium [21].

Whereas the role of the Gla domain in soluble blood coagulation proteins has been largely elucidated, its role in the function of gas6 is largely unknown. Mutant gas6 molecules with the Gla domain deleted can still bind Axl in solution [33] as the receptor-binding portion of gas6 resides in the C-terminal steroid hormone-binding globulin-like domain [34]. This observation notwithstanding, there is compelling evidence for a critical role of γ-carboxylation in the functional properties of gas6. Decarboxylated rat gas6 cannot stimulate Axl phosphorylation in CHO cells [35]. Gas6 binding to a human osteosarcoma cell line is calcium-dependent, indirectly implying a role for the Gla domain [36]. Gas6's mitogenic effect on vascular smooth muscle is abrogated by warfarin, an inhibitor of γ-carboxylation [37]. Gla is also indispensable for the mitogenic activity of gas6 on mesangial cells [38], gas6-mediated growth of cardiac fibroblasts [39] and in gas6–Axl-mediated protection of vascular smooth muscle from hydrogen peroxide-induced apoptosis [21].

This paper addresses the importance of γ-carboxylation for gas6 binding to phospholipid membranes and its cell survival function, with particular emphasis on human endothelial cells.

MATERIALS AND METHODS

Materials

A hemagluttinin-gas6 (gas6-HA) cDNA in pLXSN was obtained as a gift from Dr Edison Liu, the National Cancer Institute, Bethesda, MD, USA. An antibody that recognizes γ-carboxyglutamic acid was purchased from American Diagnostica Inc. (Grenwich, CT, USA). A monoclonal antibody to human gas6 was obtained from R & D Systems Inc. (Minneapolis, MN, USA).

Production of carboxylated and decarboxylated gas6

The gas6-HA cDNA was removed from pLXSN using the restriction enzyme EcoR1and cloned into pCDNA3 (Invitrogen). Human Embryonic Kidney (HEK) 293 cells were stably transfected with pCDNA3-gas6-HA. Cell clones expressing high levels of human gas6 protein were selected in G418 (1 mg/ml) and expanded in DMEM supplemented with 5% fetal bovine serum (Invitrogen), vitamin K (5 μg/ml), penicillin (50 u/ml) and streptomycin (50 μg/ml). Human gas6 was then purified from serum-free supernatant of gas6-transfected cells by immunoaffinity chromatography using a monoclonal antibody to human gas6 (R&D Systems). Decarboxylated gas6 was purified from cells initially grown in warfarin (0.33 μg/ml, Dupont Pharma) for 48 hours and then in media devoid of vitamin K.

Cells

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords, cultured and used between passages 3 and 6 for serum starvation experiments as described previously (28).

Transfection of NIH3T3 fibroblasts

NIH3T3 were transiently transfected (Fugene, Roche) with pCDNA3-gas6-HA or pCDNA3 alone and grown in media containing vitamin K or warfarin. After 48 hours, transfected cells were serum starved in the presence of vitamin K or warfarin for an additional 48h. The cells were then harvested, washed twice with PBS (Gibco Invitrogen) and stained with propidium iodide (Sigma). Apoptosis was quantified by sub-G_1 analysis as described below.

Detection and quantification of apoptosis in HUVECs

Apoptosis was quantified by flow cytometry (Becton Dickinson) after staining with propidium iodide (Sigma) and enumerating the hypodiploid apoptotic cells in the sub-G1 fraction.

Western blot analysis and Immunoprecipitation

HUVECs were grown in 100 mm dishes. After reaching 70% confluency (3×10^6 cells), they were treated with 100 or 400 ng/ml of carboxylated or decarboxylated human recombinant gas6 as indicated. HUVECs were trypsinized, pelleted at 300 x *g*, and then lysed by the addition of 500 µl lysis buffer {50 mM NaF, 50 mM Tris -HCl ,pH 7.5, 1%

Igepal, 0.1 mM EDTA (pH 8.0), 150 mM NaCl, 10 mM $NaPO_4$, 10% glycerol, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride plus Complete Protease Inhibitor Cocktail (Roche)}. Samples comprising equal amounts of total protein were immunoprecipitated by an anti-Axl antibody (Santa Cruz) followed by Western blot analysis of tyrosine phosphorylated proteins with a monoclonal anti-phosphotyrosine antibody (Transduction Laboratories). Akt phosphorylation was detected by Western Blot analysis using anti-pAkt and anti-Akt (Cell Signaling Technology) rabbit polyclonal antibodies as previously described (28).

Preparation of Phospholipid Vesicles

Small unilamellar phospholipids vesicles (PC:PS:dansyl PE, 65:25:10) were prepared by the method of Barenholz (40).

Measurement of Phospholipid Binding by Fluorescence Energy Transfer

3 μM small unilamellar phospholipid vesicles (PC:PS:dansyl-PE, 65:25:10) in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$ were placed in a quartz fluorescence cuvette (10 mm pathlength). Carboxylated or decarboxylated gas6 were added at the indicated concentrations and the emission at 560 nm after excitation at 280 nm was measured on an LS-55 Luminescence Spectrometer (Perkin Elmer Instruments). Dissociation constants (K_D) were calculated using a bimolecular equilibrium model as described previously (41).

RESULTS

Production of recombinant human gas6

In order to study the role of γ-carboxylation in gas6 function, we produced recombinant decarboxylated gas6 and compared its biochemical and biological properties to carboxylated gas6. We transfected HEK 293 cells with a cDNA encoding human gas6, grew these cells in the presence of vitamin K and then harvested the supernatant. To produce decarboxylated gas6, gas6-transfected HEK 293 cells were initially grown in media containing warfarin for 48 h and then in media devoid of vitamin K. Gas6 was purified from harvested supernatant by immunoaffinity chromatography using a monoclonal antibody that recognizes both carboxylated and decarboxylated gas6 (Fig. 3-1 A). As shown in Fig. 3-1 B, gas6 grown in the presence of vitamin K is γ-carboxylated whereas gas6 grown in the absence of vitamin K is not carboxylated.

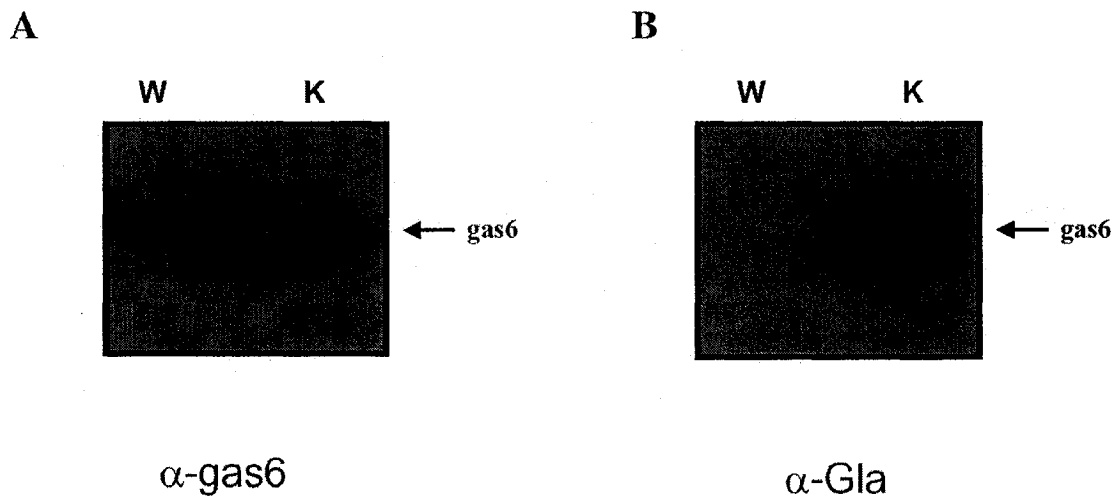

Figure 3-1. γ-carboxylation of gas6 is inhibited by warfarin. HEK 293 cells were stably transfected with pCDNA3-gas6-HA and grown in media containing 5 μg/ml vitamin K (K) or 1 μM warfarin (W). The cells were then serum starved for 48 hours after which growth medium was collected, subjected to SDS-PAGE and immunoblotted with antibodies against (A) gas6 and (B) γ-carboxyglutamic acid.

Carboxylated gas6 binds to phospholipids membranes

One of the principal biophysical properties of the Gla domain in the vitamin K-dependent γ-carboxylated coagulation proteins is the mediation of binding of these proteins to phosphatidylserine (PS)-containing phospholipid membranes [44]. To investigate this property in gas6, we examined the binding of both carboxylated and decarboxylated gas6 to phospholipid membranes using the technique of fluorescence energy transfer. A dansyl group, whose excitation and emission spectra are 340 and 560 nm respectively, was incorporated into phospholipid membranes. As gas6 binds to these dansyl-containing membranes, its tryptophan residues (emission at 340 nm after excitation at 280 nm) will excite the neighboring dansyl group in the membrane resulting in a dansyl emission at 560 nm. Therefore, after excitation at 280 nm, emission at 560 nm is a measure of gas6 binding to dansyl-containing phospholipid membranes. As shown in Fig. 3-2, carboxylated gas6 binds to PS-containing phospholipid membranes in a calcium-dependent manner with a K_D of 71 ± 9 nM. This binding is reversible in the presence of excess EDTA (not shown). No binding of decarboxylated gas6 to phospholipid membranes is detected.

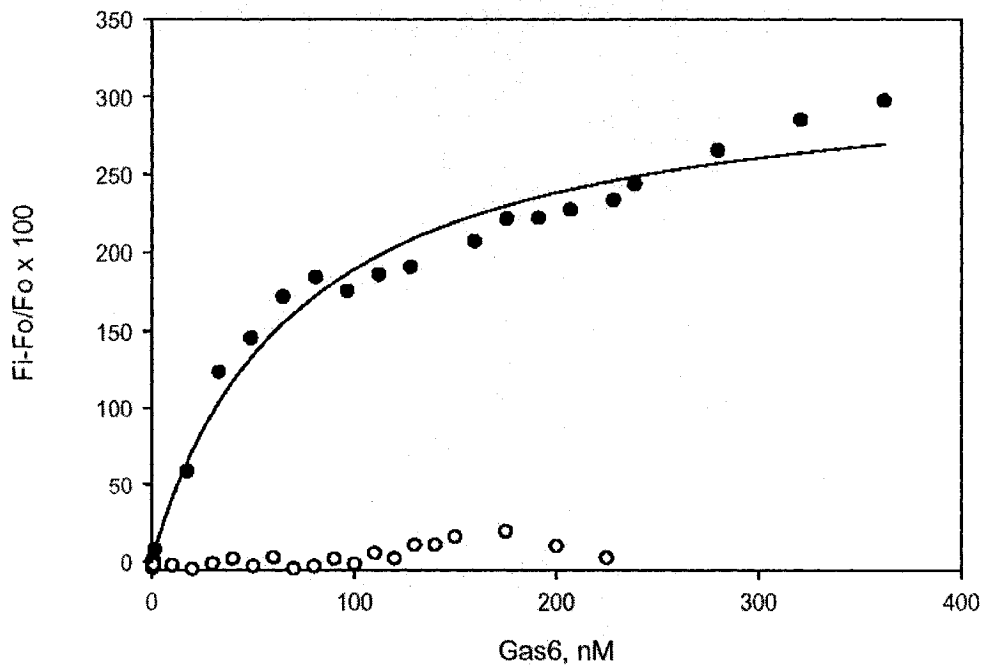

Figure 3-2. Carboxylated gas6 binds to phospholipid membranes. The binding of gas6 to phospholipid vesicles was measured by fluorescence energy transfer on an LS-55 Luminescence Spectrometer (Perkin Elmer). The indicated concentrations of carboxylated gas6 (●) or decarboxylated gas6 (○) were added to 3 μM phospholipid vesicles (PC:PS:dansyl PE, 65:25:10) in 5 mM $CaCl_2$, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4 as described in the Materials and Methods. Emission was monitored at 560nm after excitation at 280nm. The dissociation constant, K_D, is 71 ± 9 nM, and the binding is reversible with EDTA.

Role of gas6 carboxylation in protection from apoptosis in NIH 3T3 fibroblsats

To study the role of γ-carboxylation on the biologic effects of gas6, we examined the effect of the γ-carboxylation inhibitor warfarin on gas6-mediated cell survival using NIH3T3 fibroblasts as a model system. NIH3T3 fibroblasts were chosen as they are amenable to standard transfection methodologies for introducing gas6 DNA into cells. To measure cell survival, transfected cells were induced, through serum starvation, to undergo apoptosis. As shown in Fig. 3, gas6-transfected NIH3T3 fibroblasts are protected from serum starvation-induced apoptosis and warfarin abrogates this antiapoptotic effect.

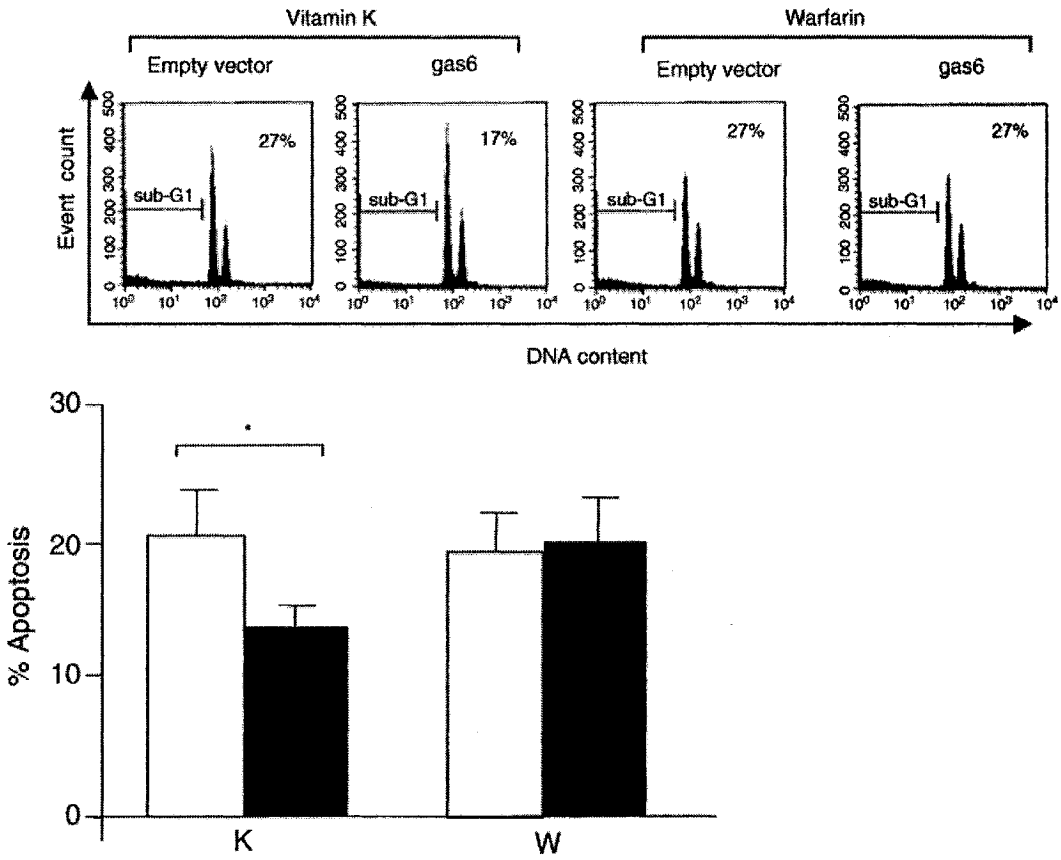

Figure 3-3. Carboxylated gas6 protects NIH3T3 fibroblasts from serum starvation-induced apoptosis. NIH3T3 fibroblasts were transfected with empty vector (□) or gas6 (■) and serum starved in presence (W) or absence (K) of warfarin. The percentage of cells undergoing apoptosis by sub-G1 analysis was measured by flow cytometry as described in Materials and Methods. Data shown are mean values ± SEM of 3 independent experiments (* $p<0.02$).

Role of gas6 carboxylation in protection of HUVEC from serum depravation induced apoptisis

In order to examine the importance of carboxylation on gas6-mediated survival of endothelial cells, purified recombinant carboxylated and decarboxylated gas6 were used to rescue endothelial cells from apoptosis. HUVECs were induced to undergo apoptosis by serum starvation for 48 h. In the presence of carboxylated gas6, there is a marked protection from apoptosis (Fig. 3-4). Thus, as shown in Fig. 3-4A, serum-starved endothelial cells are elongated and less numerous than either control serum-replete cells or serum-starved cells treated with carboxylated gas6. Whereas carboxylated gas6 maintains the confluence and the polygonal morphology of live endothelial cells, decarboxylated gas6-treated endothelial cells are morphologically similar to serum-starved cells in that they are undergoing the aforementioned apoptotic changes.

The nuclear morphologic changes are confirmed with DAPI staining as shown in Fig. 3-4B. Nuclei from HUVECs that have been subjected to serum starvation or serum starvation in the presence of decarboxylated gas6 are few in number and pyknotic. In contrast, serum-starved cells treated with carboxylated gas6 are similar to HUVECs that are serum replete as indicated by the micrographs showing large non-apoptotic nuclei (Fig. 3-4B). These results are confirmed quantitatively by counting the percentage of apoptotic nuclei by DAPI staining and using flow cytometry via enumerating the number of cells in the sub-G1 fraction, representing hypodiploid apoptotic cells (Fig. 3-4C). Whereas gas6 protects HUVECs from serum starvation-induced apoptosis [28–30], decarboxylated gas6 is unable to do so. Figure 4D shows results with Annexin V staining

and is similar to results in Fig. 3-4C, namely that carboxylated but not decarboxylated gas6 protect HUVECs from serum starvation-induced apoptosis. These results are consistent with those obtained with NIH3T3 fibroblasts (see Fig. 3-3) and support the conclusion that γ-carboxylation is necessary for the antiapoptotic effect of gas6.

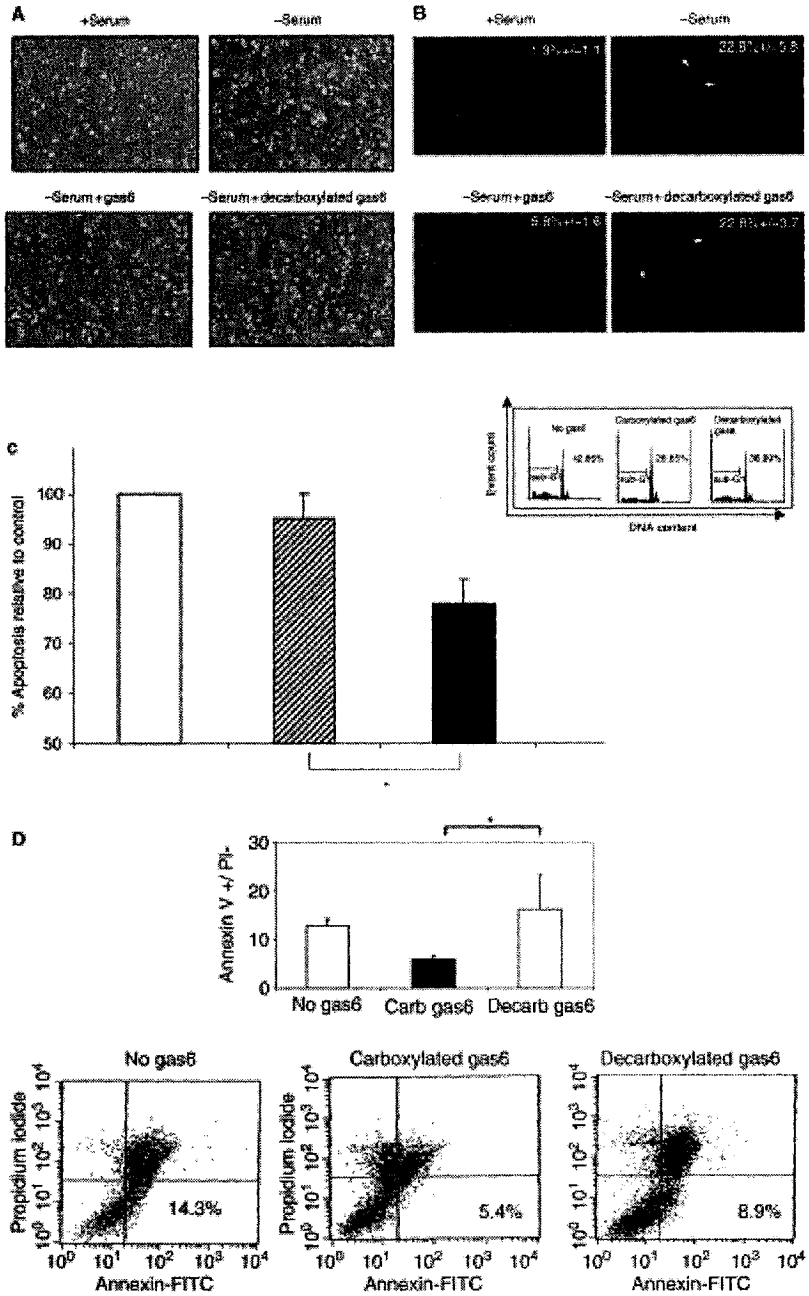

Figure 3-4. γ-carboxylation of gas6 is necessary for the protection of HUVECs from serum starvation induced apoptosis. HUVECs were serum starved, as described in Materials and Methods, in the presence of 100 ng/ml carboxylated gas6 or 100 ng/ml decarboxylated gas6. A, Phase contrast micrographs of HUVECs in culture. B, DAPI staining showing apoptotic nuclei (arrowhead). C, Carboxylated gas6 protects HUVECs from serum starvation-induced apoptosis. HUVECs were serum starved for 48 hours in the absence of gas6 (□) or presence of 100ng/ml of carboxylated (■) or decarboxylated gas6 (▨). The percentage of cells undergoing apoptosis was measured by flow cytometry using sub-G1 analysis and D, Annexin V staining. Data shown are mean values ± SEM of 4 independent experiments (* $p<0.01$)

Importance of γ-carboxylation for Axl phosphorylation and Akt activation

Gas6 mediates its antiapoptotic effect through activation of the receptor tyrosine kinase, Axl. We have previously shown that gas6 protects endothelial cells from apoptosis utilizing classical intracellular signaling pathways such as Akt activation through PI_3 kinase [28]. Figure 3-5A indicates that carboxylated gas6 is able to phosphorylate Axl in HUVECs whereas decarboxylated gas6 cannot. Furthermore, as shown in Fig. 3-5B, carboxylated but not decarboxylated gas6 leads to Akt phosphorylation demonstrating that only carboxylated gas6 can result in Akt activation.

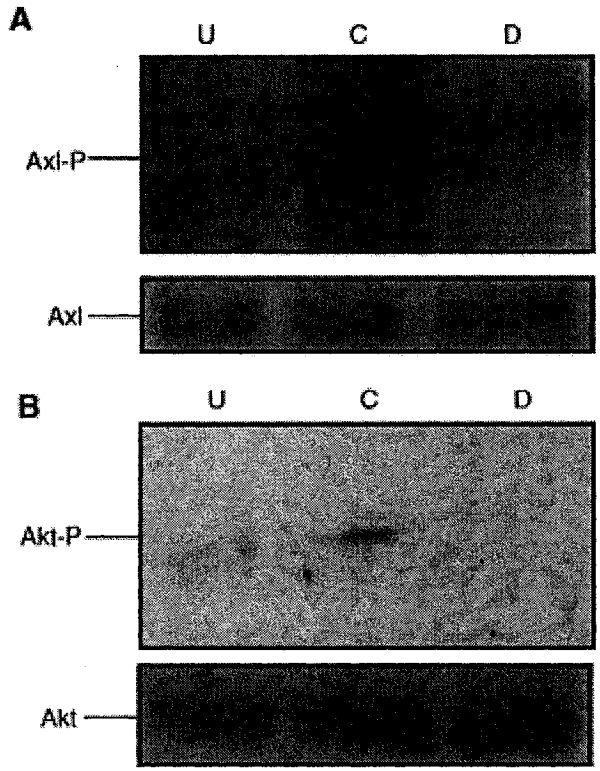

Figure 3-5. γ-carboxylation is necessary for Axl phosphorylation and Akt activation. A. HUVECs were either untreated (U) or stimulated with 400 ng/ml carboxylated (C) or decarboxylated (D) gas6 for 10 minutes, lysed and immunoprecipitated with a polyclonal anti-Axl antibody as indicated in Materials and Methods. The immunoprecipitate was then subjected to SDS-PAGE and developed, by Western analysis, with an antiphosphotyrosine antibody or an anti-Axl antibody. B. HUVECs were serum starved for 4 hours in the presence of 100 ng/ml of carboxylated (C) or decarboxylated (D) gas6 or left untreated (U) as indicated. Cell lysates were then subjected to SDS-PAGE and probed with both an anti-pAkt and anti-Akt antibody

DISCUSSION

The vitamin K-dependent blood clotting proteins contain γ-carboxyglutamic acid in order to allow these proteins to bind to negatively charged phospholipid membranes [44]. Prior to the present study, it was not known whether γ-carboxylation imparts phospholipid-binding properties on gas6. Furthermore, the importance of phospholipid binding for gas6/receptor interactions was not clear and whether γ-carboxylation is relevant for gas6-mediated protection of cells from apoptosis had not been addressed, as had been shown for the mitogenic effect of gas6 [39].

In the present study, we show directly that the Gla domain is essential for gas6/phospholipid interaction. Although Nagano *et al.* [45] reported previously that gas6 binds to phosphatidylserine immobilized on a microtitre plate, the present experiments (Fig. 2) demonstrate binding to phospholipid membranes comprising a mixture of PS, phosphatidylcholine (PC), and phosphatidylethanolamine (PE), which likely recapitulates biological membrane binding. Indeed, for the assembly of the tenase complex, both PS and PE are important [46]. The affinity of gas6 for these phospholipid vesicles is 71 nM, similar to that of other vitamin K-dependent blood coagulation proteins including protein S [47], the vitamin K-dependent blood coagulation protein with the greatest homology to gas6.

To study the role of γ-carboxylation in cell survival, we demonstrate that warfarin prevents the survival-mediated effect of gas6 on NIH3T3 fibroblasts (Fig. 3). The effect of warfarin is presumably mediated through inhibition of γ-glutamyltransferase [4] with subsequent production of decarboxylated gas6. Similar data in mesangial cells have been

reported by Nagai *et al.*, however, the possibility that warfarin has other effects in the complex biological systems being examined cannot be discounted. For example, in preliminary studies (not shown), HEK 293 cells do not survive in culture for prolonged periods in the presence of warfarin. Accordingly, we decided to explore biological models that rely only on the carboxylation state of gas6 by utilizing purified carboxylated or decarboxylated gas6.

Although other studies have examined the role of γ-carboxylation in gas6 in fibroblasts [39] and the role of warfarin on mesangial cell proliferation [16,48], the present study provides direct evidence that the Gla domain is necessary for cell survival. Not only is carboxylation required for gas6 protection of endothelial cells from apoptosis, but also intracellular activities such as Axl phosphorylation and Akt activation are abrogated when gas6 is decarboxylated. Therefore, the carboxylation state of the ligand is crucial for the activation of Axl. This contrasts with findings by Mark *et al.* [33] showing that gas6 lacking a Gla domain is still able to activate Rse. One plausible explanation to account for the difference between decarboxylated gas6 and gas6 lacking an entire Gla domain is the nature of the tertiary structures of Gla domains in general. For example, the solution structure of the Gla domain of factor IX reveals that Gla domains, in the presence of calcium, are highly ordered and compact structures [49]. In the absence of negatively charged γ-carboxyglutamic acid side chains binding calcium, these structures become highly disordered [50]. Such a disordered structure in the Gla domain of gas6 may interfere with proper gas6–Axl interactions thereby abrogating the biological effect of gas6.

Other soluble γ-carboxylated proteins have been shown to interact with membrane-bound receptors. The Gla domain of factor VIIa (FVIIa) interacts directly with tissue factor, a type I membrane protein. However, the Gla domain in FVIIa is only one of several contact sites within FVIIa that interact with tissue factor [51]. Another γ-carboxylated protein, protein C, binds to its receptor on endothelial cells, the endothelial cell protein C receptor (EPCR), primarily via its Gla domain [52]. In contrast to FVIIa and protein C, the Gla domain is not important for the direct binding of gas6 to Axl [33]. Therefore, the manner by which the Gla domain imparts functionally relevant interactions of gas6 with membrane-bound Axl is not clear. As discussed above, the Gla domain of gas6 may alter the tertiary structure of gas6 such that the association of its C-terminal receptor-binding region to Axl is promoted [53]. Alternatively, the association of the C-terminus of gas6 with Axl may depend on Gla-mediated anchoring of gas6 to the membrane. This latter hypothesis is supported by the observation that the affinity of gas6 for its receptor differs depending on whether the receptor is in solution or in a cell membrane [33,37].

In conclusion, the results presented in this paper provide a direct demonstration for the requirement of γ-carboxylation in the binding of gas6 to membranes and show the importance of γ-carboxylation for gas6-mediated survival of endothelial cells. Furthermore, these data demonstrate a unique role for γ-carboxylation in endothelial physiology and imply a novel mode of action and use of warfarin in vascular biology beyond its known effect in anticoagulation.

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Chapter 4

GENERAL DISCUSSION

4.1 Gas6 is an important mediator of endothelial cell survival

Gas6 is a member of the vitamin K-dependent family of proteins and is a ligand for the receptor tyrosine kinase, Axl. The relative abundance of these two proteins in various cell systems and the unique translational modification of gas6 has facilitated the discovery of the many different roles these proteins play. Gas6-Axl interactions have been implicated in several different cell systems; in vascular smooth muscle, gas6 mediates smooth muscle cell proliferation (Nakano et al., 1995); in the kidney, gas6-Axl interactions are important for mesangial cell proliferation (Yanagita et al., 2001), nephrotoxic glomerular injury (Yanagita et al., 2002) and diabetic nephropathy (Nagai, et al., 2003); in bone, gas6-Axl interactions upregulate osteoclast function (Katagiri et al., 2001), in the central nervous system, gas6 protects neurons from amyloid-induced apoptosis (Yagami et al., 2002); in the eye, gas6 mediates outer retinal pigment epithelial function (Hall et al., 2001); and finally homozygous null gas6 mice have a platelet dysfunction that protect mice against lethal intravascular thrombosis (Angelillo-Scherrer et al., 2001). In endothelial cells, gas6-Axl interactions have been reported to play an important role in cell survival (O'Donell et al., 1999) and proliferation (Healy et al., 2001) but there is little evidence of the signalling pathways involved.

The study described in Chapter 2 provides clear evidence that gas6 acts as a survival factor in endothelial cells (EC) and describes the signaling pathway involved in gas6-mediated protection from apoptosis. Endothelial cell survival is linked to numerous pathological conditions including tumor growth and metastasis, rheumatoid arthritis, proliferative diabetic retinopathy, atherosclerosis, and post-ischemic vascularization of the myocardium (Isner and Losordo, 1999). Thus, understanding the cell signalling events that lead to the inhibition of apoptosis due to gas6-Axl interactions in endothelial cells may contribute to a better understanding of these conditions.

Programmed cell death or apoptosis is a highly regulated process, initiated by the presence of apoptotic factors and absence of growth factors (chapter 1). Gas6 acts as a growth factor in several cell types. In endothelial cells, gas6 displays an anti-apoptotic effect through the activation of its receptor Axl. Once activated, Axl induces PI3 kinase and Akt activation in NIH3T3 fibroblasts, known anti-apoptotic inducers. Similarly to NIH 3T3 fibroblasts, this study shows that gas6 has an antiapoptotic effect in human umbilical endothelial cells (HUVEC) (chapter 2) and that low concentrations of gas6 protect these cells form serum starvation for up to 72 hours. Furthermore, like the other growth factors, gas6 protects endothelial cells through PI3 kinase activation and subsequent Akt phosphorylation as demonstrated by dominant negative Akt studies and the PI3 kinase inhibitor wortmannin. A number of different growth factors have been shown to rapidly activate Akt via PI3-kinase activation, such as platelet-derived growth factor, epidermal growth factor, bFGF, insulin, and insulin-like growth factor 1 (Kohn et al., 1995; Franke et al., 1995; Burgering et al., 1995; Andjelkovich et al., 1996 ; Harrington et al., 1994)

Other studies have examined Akt phoshorylation in endothelial cells. For example, VEGF, an important regulator of vascular development, mediates its survival signal through the PI3 kinase/Akt transduction pathway (Gerber et al., 1998).More specifically, decreased levels of VEGF-dependent survival are observed when cells were exposed to wortmannin or LY 294002, two potent PI3-kinase inhibitors. PI3-kinase activation increases the intracellular amounts of phosphatidylinositol-3,4,5-bisphosphate and phosphatidylinositol-3,4,5-triphosphate, which positively regulate Akt by binding to the pleckstrin homology domain of Akt (chapter1). Thus, Akt activation by growth factors requires PI3-kinase activity. In our study, gas6 induces PI3 kinase activation leading to subsequent Akt phosphorylation which is sustained for up to 72 hours. After its activation, Akt transduces signals that regulate various biological processes including apoptosis, gene expression, and cellular proliferation. Thus, the wide range of biological effects of activated Akt means that it may phosphorylate numerous target proteins. To date, few of them have been identified, including Gsk-3β (Cross et al., 1995), Bad (Datta et al., 1997; del Peso et al., 1997), pro-caspase9 (Cardone et al., 1998), CREB (Du and Montminy, 1998) Erk (Cenni et al., 2003). Akt also affects the transcriptional response to apoptotic stimuli by affecting the Forkhead transcription factors (Burgering and Medema, 2003) and p53 activity. In addition Akt affects the activity of IκB (Buja et al., 1993),a regulator of NF-κB transcriptional activity.

In our first study we demonstrate for the first time that gas6 affects these targets in endothelial cells through activation of Akt. More specifically, we show that activation of Akt in response to gas6-Axl interaction results in increased levels of the anti-apoptotic protein Bcl-2 as well as decreased levels of activated caspase 3. The results are consistent

with a key role of this protease described in several other cell systems. For example, shear stress promotes endothelial cell survival and does so by reducing caspase 3 activity (Kane et al., 1999). Furthermore, we also demonstrate that gas6 is able to induce NF-κB phosphorylation as well as a rapid increase in NF-κB transcriptional activity (Figure 4-1) thus playing a role in the transcriptional control of apoptosis. Taken together, results from our first study indicate that gas6 acts as an important mediator of endothelial cell survival during serum stress through activation of classical antiapoptotic pathway, namely, Akt phosphorylation, NF-κB activation, increased Bcl-2 protein expression, and a reduction in caspase 3 activation.

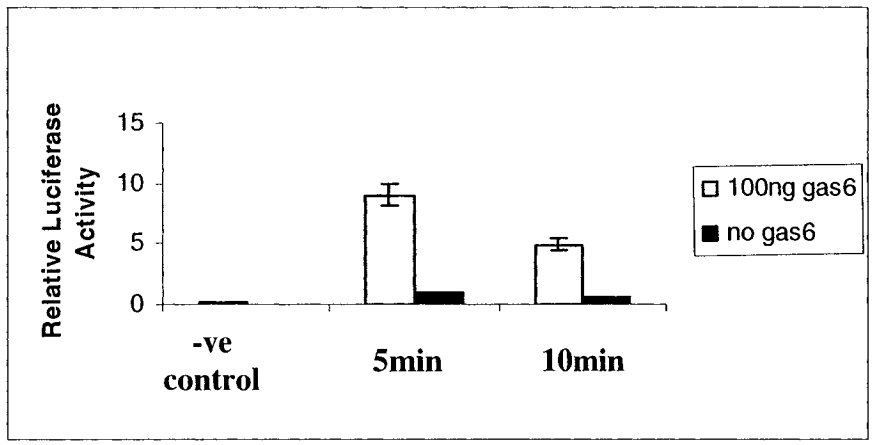

Figure 4-1. Transcriptional activation of NF-κB by gas6. Cells were transfected with NF-κB luciferase construct, arrested overnight at 0.5% FBS and serum starved for indicated times in the presence or absence of 100ng/ml of gas6. Negative control sample was not transfected. Data shown are mean ± SEM of 3 independent experiments (*$p<0.05$).

The importance of gas6-Axl interactions in endothelial cell physiology is evidenced in gas6-mediated protection of HUVECs and human pulmonary artery endothelial cells from serum starvation (Healy et al., 2001; O'Donnell et al., 1999). Recent studies of endothelial cell physiology have demonstrated that the endothelium undergoes apoptosis. Thus, information about mechanisms underlying the regulation of endothelial cell survival is fundamentally important for understanding angiogenesis and vascular remodeling, crucial processes in a wide variety of disease states ranging from atherosclerosis to tumor metastasis and may provide a basis for rationale drug design aimed at manipulating this pathway under pathological conditions.

4.2. The role of Gla domain of gas6 in endothelial cell survival

A most intriguing feature of gas6 as a mediator of cell survival is its unusual posttranslational modification, γ-carboxylation. Whereas the role of the γ-carboxyglutamic acid domain (Gla) domain in soluble blood coagulation proteins has been largely elucidated, its role in the function of gas6 is largely unknown. Thus, understanding the role of Gla domain of gas6 in gas6-Axl interaction is of fundamental importance since γ-carboxylation may influence the anti-apoptotic property of gas6-Axl interaction.

The Gla domain of gas6 is located on its N-terminus whereas the receptor binding domain is located on the C-terminus. Recently, the crystal structure of gas6 C-terminus receptor binding structure has been solved, precisely outlining hydrophobic residues necessary for Axl receptor binding (Sasaki et al., 2001). Gas6 mutants lacking the Gla domain can still bind Axl receptor in solution (Mark et al., 1999). However, there is

compelling evidence suggesting that γ-carboxylation plays a critical role in the functional property of gas6. This evidence shows that decarboxylated gas6 cannot stimulate CHO cells expressing Axl (Tanabe et al., 1997). Furthermore, the importance of the Gla domain is indirectly implied in a study that demonstrates that gas6 binding to a human osteosarcoma cell line is calcium dependant (Nakano et al., 1996). As well, gas6's mitogenic effect on vascular smooth muscle is inhibited by warfarin, an inhibitor of γ-carboxylation (Nakano et al., 1997).

In mesangial cells, the Gla domain of gas6 is indispensable for its mitogenic activity (Yangita et al., 2002; Yangita et al., 1999). Computer modeling of the Gla domain of gas6 suggests that it should be capable of binding to negatively charged phospholipid membranes (Perera et al., 1997). Alternatively, a proteolytically processed splice variant of gas6 in which Gla domain is removed suggests possible regulatory role of Gla domain (Goruppi et al., 1997; Marcandalli et al., 1997). Thus, the above evidence suggests an importance for the Gla domain in the functional properties of gas6.

The Gla domain requires calcium for mediating protein-lipid and protein-protein interactions. This has been studied in the vitamin K dependant blood coagulation proteins (Freedman et al., 1996 & 1995; Blostein et al., 2003; Borowski et al., 1986). In the presence of calcium, vitamin K dependant proteins undergo a conformational change in their tertiary structure (Nelsestuen, 1976). This conformational change is necessary for allowing Gla domain proteins to bind to phospholipid membranes. Based on the computer model, the γ-carboxyglutamic acid residues that form the Gla domain located on the N-terminus of gas6 directly bind calcium ions thereby enabling them to interact tightly with anionic phospholipid surfaces, such as those displayed by cells undergoing

apoptosis (Perera et al., 1997). Experimentally, it has been demonstrated that calcium is necessary in the binding of gas6 to phosphatidylserine (PS) bound to a microtitre plate (Nakano et al., 1997). However, the calcium requirement for binding of gas6 to PS has not been measured in cultured cells.

Our study shows, for the first time, that, in the presence of calcium, only carboxylated but not decarboxylated gas6 binds to negatively charged phospholipid membranes. Thus, we show directly that Gla domain of gas6 is necessary for binding to negatively charged phospholipids membranes in the presence of calcium (chapter 3). The affinity of gas6 for these phospholipids is 71 nM, similar to that of other vitamin K-dependent blood coagulation proteins including protein S (Walker, 1984), the vitamin K-dependent blood coagulation protein with the greatest homology to gas6.

The role of the Gla domain in soluble blood coagulation proteins has been extensively studied; however, its role in the function of gas6 is still largely unknown. Uncarboxylated rat gas6 cannot stimulate Axl phosphorylation in CHO cells (Tanabe et al., 1997). Further evidence of a critical role of γ-carboxylation in functional properties of gas6 is provided in the study that demonstrates that only fully γ-carboxylated gas6 was able to exert its protective properties in serum starved cells (Stenhoff et al., 2004). This study shows that in cardiac fibroblasts the mitogenic capacity of gas6 in its uncarboxylated form was abolished and was unable to induce Axl tyrosine phosphorylation. Similarly, our study shows that in HUVECs, only carboxylated gas6 is able to induce Axl phosphorylation whereas decarboxyalated gas6 cannot. Additional inhibition experiments using an anti-Axl antibody to inhibit Axl activation clearly demonstrate that both carboxylated and decarboxytlated gas6 bind equally to the Axl

receptor (Figure 4-2). These results indicate that γ-carboxylation of gas6 does not affect its ability to bind Axl but it unquestionably influences gas6 ability to phosphorylate Axl (Chapter 3).

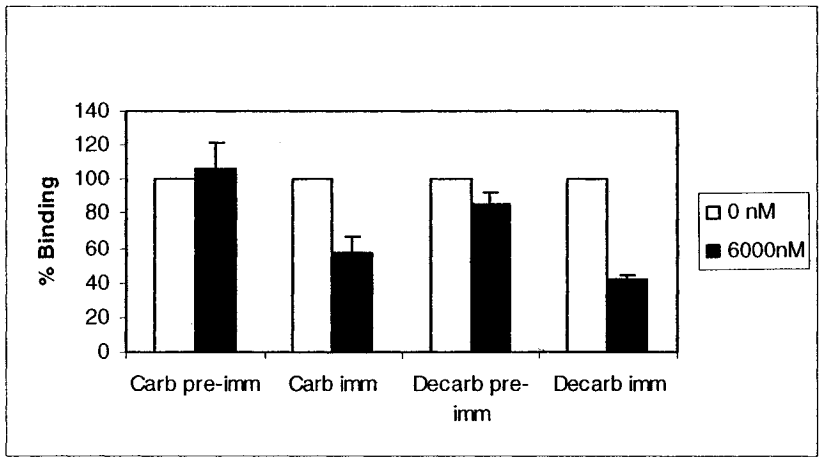

Figure 4-2. The binding of gas6 to HUVECs is inhibited by an antibody to Axl. HUVECs were incubated in the presence of a control immunoglobulin (□) or with an antibody to the extracellular domain of Axl (■) and the binding of fluorescein labelled-carboxylated gas6 or fluorescein labelled-decarboxylated gas6 was measured by flow cytometry. Data shown are mean ± SEM of 3 independent experiments. (*$p<0.05$, **$p<0.01$).

Furthermore, we demonstrate that γ-carboxylation of gas6 affects molecules downstream of Axl, namely, Akt. Our study indicates that only fully carboxylated gas6 is able to induce Akt phosphorylation (Chapter 3). Therefore, we clearly demonstrate that only the carboxylated form of gas6 is able to protect endothelial cells from apoptosis through Axl phosphorylation and Akt activation whereas decarboxylated gas6 cannot. Additional competition experiments confirm these findings by demonstrating that

decarboxylated gas6 is able to effectively compete with and inhibit the survival effect of carboxylated gas6. (Figure 4-3).

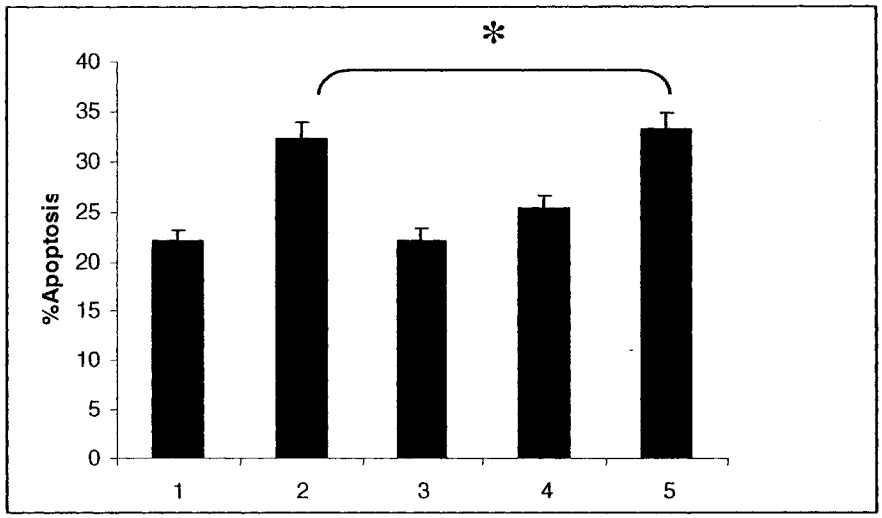

Figure 4-3. Excess of decarboxylated gas6 prevents carboxylated gas6 form protection from apoptosis. (1) 100ng/ml carboxylated gas6, (2) 100 ng/ml of decarcarboxylated gas6, (3) 100ng/ml of carboxylated gas6 and 10ng/ml of decarboxylated gas6, (4) 100ng/ml of carboxylated gas6 and 100ng/ml of decarboxylated gas6, (5) 100ng/ml of carboxylated gas6 and 1000ng/ml of decarboxylated gas6. The percentage of cells undergoing apoptosis was measured by flow cytometry using sub-G1 analysis. Data shown are mean values ± SEM of 3 independent experiments (* $p<0.02$).

These results clearly demonstrate importance of Gla domain in gas6 function and confirm the proposed hypothesis that gas6 is only fully biologically active in its γ-carboxylated form. To further assess the importance of Gla domain of gas6 in antiapoptotic function we demonstrate that Annexin V, a protein that has a high affinity for exposed phosphatidylserine (PS) on apoptotic cells, can inhibit the survival effect of properly carboxylated gas6, thus implicating the importance of Gla domain (Figure 4-4). Moreover, additional results using soluble PS to compete for binding of carboxylated gas6 with membrane bound PS in apoptotic cells confirms previous results indicating that in the presence of soluble PS, gas6 is unable to protect endothelial cells from serum-

stress induced apoptosis (Figure 4-5). Taken together these results clearly demonstrate that the Gla domain of gas6 is indispensable for its antiapoptotic function in endothelial cells.

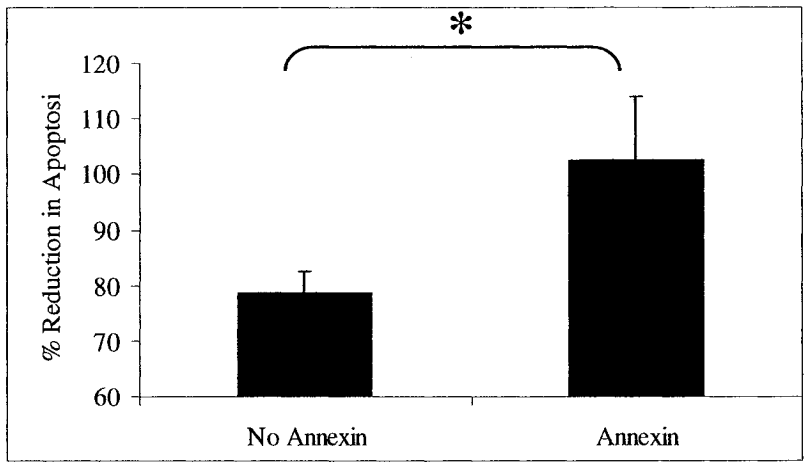

Figure 4-4. The survival effect of gas6 is inhibited by annexin V. HUVECs were serum starved in the presence of 100 ng/ml gas6 and in the absence or the presence of 5 μg/ml of annexin V. The percentage of cells undergoing apoptosis was measured by flow cytometry using sub-G1 analysis. Data shown are mean ± SEM of 4 independent experiments. (*$p<0.01$).

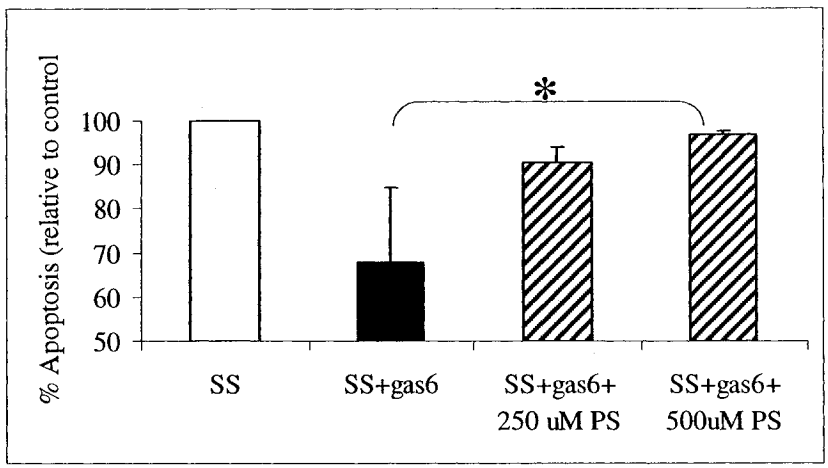

Figure 4-5. The survival effect of gas6 is inhibited by soluble phosphatidylserine. HUVECs were serum starved in the absence or the presence of 100ng/ml gas6 or in the presence of gas6 and soluble phosphatidylserine (PS) at the indicated concentrations. The percentage of cells undergoing apoptosis was measured by flow cytometry using sub-G1 analysis. Data shown are mean ± SEM of 3 independent experiments. (*$p<0.2$).

In addition, the current study strengthens the hypothesis that vitamin K-dependant processes are important not only for the production of proteins involved in blood coagulation but also for the production of gas6. Vitamin K reactions may be of additional importance in development and disease processes involving cell proliferation and survival (Saxena et al., 2001). For instance, Saxena et al showed that in chick embryos, vitamin K levels are crucial at determining level of tyrosine phosphorylation and subsequent embryo development. Thus, manipulation of the Gla domain of gas6 may lead to the development of novel therapies.

One way to manipulate Gla domain is by warfarin, an inhibitor of gamma-carboxylaion. Warfarin inhibits carboxylation by affecting a warfarin sensitive enzyme, vitamin K epoxide reductase (VKOR). This recently cloned enzyme is responsible for

recycling vitamin K into its active form, a necessary cofactor for the γ-carboxylation reaction (Wajih et al., 2004). Since use of warfarin may have other effects in complex biological system, manipulation of Gla domain by silencing VKOR may not only provide new avenues in regulation of cell growth or inhibition of apoptosis by gas6 but may also may significantly improve therapies where warfarin commonly used.

There are several hypotheses that can account for the role of Gla domain in imparting functionally relevant interactions of gas6 with membrane bound Axl. First the Gla domain may allow gas6 to localize the C-terminal receptor-binding portion to its receptor by binding to negatively charged phospholipid membrane. This is evidenced in studies examining the kinetics of prothrombinase complex assembly (Kirshnaswamy et al., 1988) where Gla domain plays an important role in facilitating protein-protein interaction at membrane surface. Also, protein-protein interactions mediated by Gla domain are important in the assembly of enzymatic complexes in blood coagulation (Blostein et al., 2000 & 2003). Second, the Gla may act as an adhesion molecule with C-terminus binding to its receptor on one cell and the N-terminus binding to negatively charged membrane on the neighbouring cell (Nakano et al., 1997; McCloskey et al.,1997). Third, the Gla domain may alter the conformation of gas6 thereby modulating its affinity for Axl (Tanabe et al., 1997). Even though the manner by which the Gla domain imparts functionally relevant interactions of gas6 with membrane-bound Axl is not yet clear, it is evident that this post-translational modification imparts a significant role in function of gas6.

4.3 Future Directions

Gas6 acts as antiapoptotic factor in endothelial cells and its unique post-translational modification plays an important role in this protective function. The design of experiments to show the exact mechanism of Gla domain function in the anti-apoptotic property of gas6 may prove to be very interesting. Using a purified model system comprising gas6 and extracellular and transmembrane domains of Axl (Axl-ECD/TM) reconstituted into phospholipid membranes might give insight into structural basis of the interaction between the Gla domain of gas6 and Axl. Furthermore, synthesizing a series of gas6 mutants where the Gla domain has been completely deleted or mutated, or the production of gas6/ blood coagulation protein (e.g. factor IX) chimeras with Gla domain of gas6 replaced with Gla domain of factor IX, for example, will provide further clues to delineate the mechanism of action of the Gla domain of gas6. In addition, the study outlined in Chapter 2 has demonstrated that gas6 protection of endothelial cell apoptosis is regulated by classical antiapoptoic pathways such as, PI3 kinase, Akt, Bcl-2 and caspase 3 activation. It would be of great interest to examine other genes that might be regulated during gas6-mediated survival of endothelial cells using a DNA Microarray approach and Real Time PCR. Moreover, the question of whether gas6-Axl system is involved in the protection of endothelial cells from other, more physiologically relevant apoptotic stimuli such as oxidative stress would further outline the importance of this system in vascular biology.

Another important area of gas6 investigation is its possible role in angiogenesis. Angiogenesis is crucial in tumor growth and metastasis. It involves the proliferation of endothelial cells and if gas6 has mitogenic and antipapoptotc effects for vascular

endothelium and manipulation of gas6 and its post-translational modification may have significant effects on tumor growth. Thus, models of endothelial cell physiology examining the effect of gas6 and warafarin on in vivo model of angiogenesis utilizing retinal capillary endothelial cells or on animal models of tumor metastasis would greatly contribute to assessing the angiogenic potential of gas6. Finally, emerging epidemiologic data have demonstrated that warfarin use can lower the development of malignancies (Schulman and Lindmarker, 2002). Studying gas6 biology and the importance of γ-carboxylation may provide insight for the emerging data correlating warfarin use and a lower incidence of malignancy. Therefore, the study of gas6 properties may have broad implications in both vascular and cancer biology.

4.4 Proposed model

Based on the experimental evidence provided by our studies on gas6-Axl interactions in endothelial cells, we present a hypothetical model in Figure 4-5. Gas6 binds to Axl on the surface of endothelial cells and induces Axl phosphorylation. Once phosphorylated, Axl promotes PI3 kinase activation and subsequent Akt phosphorylation. Activation of Akt leads to activation of known antiapoptotic factors, namely, Bcl-2 and NF-κB as well as inhibition of procaspase 3 cleavage to its active form. Taken together activation of these antiapoptotic proteins lead to endothelial cell survival during serum stress. However, in the presence of wortmannin this pathway is disrupted due to PI3 kinase inhibition and cells undergo apoptosis. Similarly, in the presence of uncarboxylated gas6 or warfarin, a vitamin K carboxylase inhibitor, gas6 is unable to induce Axl phosphorylation and subsequent Akt activation causing endothelial cells to undergo serum depletion induced apoptosis.

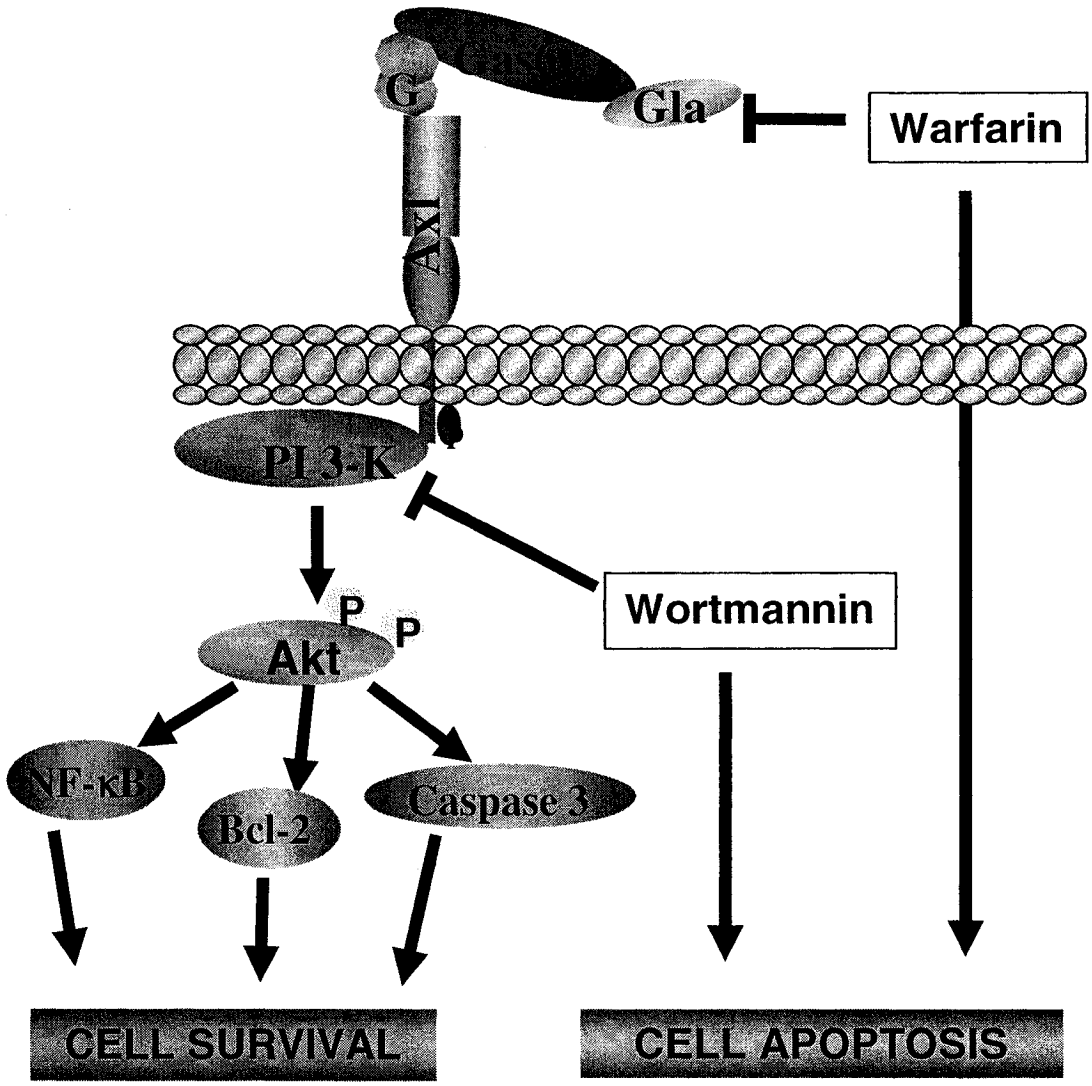

Figure 4-6. Model for gas6 endothelial cell survival pathway

APPENDIX

Methods of figures in chapter 4

Figirer 4-1. Transcriptional activation of NF-κB by gas6

HUVECs were transiently transfected with Fugene (Roche) reagent according to the manufacturer's recommendations with the p2 construct containing four NFκB binding sites followed by a luciferase gene and pRL-TK Renilla control. Transfections were done in triplicate and repeated 3 times. Cells were arrested overnight in 0.5% FBS after which they were either treated with 100 ng/ml of gas6 or left untreayed. Cells were washed with PBS without Ca^{2+} and Mg^{2+} and harvested with Passive Lysis Buffer (Promega, Madison, WI). Luciferase assays were performed using reagents from the Dual-Luciferase Reporter Assay System (Promega) and with an EG&G Berthold model Lumat LB9507 luminometer. Relative luciferase activity was calculated and reported as a ratio between firefly luciferase and *Renilla* luciferase activity.

Figure 4-2. Binding of carboxylated and decarboxylated gas6 to Axl.

HUVECs (6 x 10^5 cells/ml) were harvested in PBS with 0.02% sodium azide and resuspensed in 0.5ml of buffer. They were then incubted for 20min at 37°C in the presence of control immunoglobulin or with 6000nM of antibody to the extracellular domain of Axl. Fluorescein-labeled carboxylated and /or decraboxylated gas6 were then added to these cells for 5 min and incubated in the dark at room temperature. After incubation with gas6, cells were collected and the binding of gas6 was measured by flow

cytometry. Data shown are a mean ± SEM of 3 independent experiments. (*$p<0.05$, **$p<0.01$).

Figure 4-3. Excess of decraboxylated gas6 prevents carboxylated gas6 anti-apoptotic function.

1 x 10^6 HUVECs were plated in 100-mm tissue culture plates. Upon reaching 70% confluency, cells were placed in serum-free media in the presence or absence of either 100 ng/ml recombinant human gas6; 100 ng/ml of decarboxylated gas6; 100ng/ml of carboxylated gas6 and 10 ng/ml of decarboxylated gas6; 100ng/ml of carboxylated gas6 and 100ng/ml of uncarboxylated gas6 or 100ng/ml of carboxylated gas6 and 1000ng/ml of uncarboxylated gas6 and incubated for 48 hours. The cells were then harvested by trypsinization and washed twice with PBS (GIBCO Invitrogen). Apoptosis was quantified by flow cytometry by staining with propidium iodide (Sigma) and enumerating the hypodiploid apoptotic cells in the sub-G_1 fraction. Cells analyzed by flow cytometry (Becton Dickinson) were quantified using Cell Quest software. Data shown are mean values ± SEM of 3 independent experiments (* $p<0.02$).

Figure 4-4. Annexin V inhibits carboxylated gas6 anti-apoptotic function.

1 x 10^6 HUVECs were plated in 100-mm tissue culture plates. Upon reaching 70% confluency, cells were placed in serum-free media and incubated with 5 μg/ml of annexin V in the presence or absence of 100 ng/ml of carboxylated gas6 for 48 hours. The control dish was incubated in serum free media for 48 hours with or without 100

ng/ml of carboxylated gas6 and in the absence of Annexin V. The cells were then harvested by trypsinization and washed twice with PBS (GIBCO Invitrogen). Apoptosis was quantified by flow cytometry by staining with propidium iodide (Sigma) and enumerating the hypodiploid apoptotic cells in the sub-G_1 fraction. Cells analyzed by flow cytometry (Becton Dickinson) were quantified using Cell Quest software. Data shown are mean values ± SEM of 4 independent experiments (* $p<0.01$).

Figure 4-5. Soluble phosphatidylserine inhibits gas6's anti-apoptotic function

HUVECs (1×10^6) were plated in 100-mm tissue culture plates. Upon reaching 70% confluency, cells were placed in serum-free media and incubated with either 250 μM or 500 μM of soluble phosphatidylserine (PS) (Sigma) in the presence or absence of 100ng/ml of carboxylated gas6 for 48 hours. The cells were then harvested by trypsinization and washed twice with PBS (GIBCO Invitrogen). Apoptosis was quantified by flow cytometry by staining with propidium iodide (Sigma) and enumerating the hypodiploid apoptotic cells in the sub-G_1 fraction. Cells analyzed by flow cytometry (Becton Dickinson) were quantified using Cell Quest software. Data shown are mean values ± SEM of 4 independent experiments (* $p<0.02$).

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The present work has focused on the role of gas6-Axl interaction in vascular biology. The major contributions to original knowledge include the following:

1. Gas6-Axl interaction promotes endothelial cell survival through upregulation of Bcl2 and downregulation of caspase 3 activity
2. Post-translation modification of gas6, namely γ-carboxylation' is necessary for its biological function
3. Decarboxylated gas6 cannot protect endothelial cells from serum-starvation induced apoptosis
4. Decarboxylated gas6 binds to Axl but does not induce its phosphorylation
5. Decarboxylated gas6 does not induce Akt phosphorylation