Grb2 Is Required for the Development of Neointima in Response to Vascular Injury

Shaosong Zhang, Jie Ren, M. Faisal Khan, Alec M. Cheng, Dana Abendschein, Anthony J. Muslin

Objective—Neointima formation occurs in arteries in response to mechanical or chemical injury and is responsible for substantial morbidity. In this work, the role of the intracellular linker protein Grb2 in the pathogenesis of neointima formation was examined. Grb2 is a critical signaling protein that facilitates the activation of the small GTPase ras by receptor tyrosine kinases.

Methods and Results—Cultured rat aortic smooth muscle cells were treated with an antisense morpholino to Grb2 and these cells showed a reduced proliferative response to platelet-derived growth factor stimulation. Grb2+/− mice do not survive embryonic development. Grb2+/− mice appear normal at birth and are fertile but have defective signaling in several tissues. Cultured smooth muscle cells derived from Grb2+/− mice grew at a much slower rate than cells derived from Grb2+/+ mice. Grb2+/− and Grb2+/+ mice were subjected to carotid injury. After 21 days, Grb2+/+ mice developed robust neointima formation that, in some cases, resulted in an occlusive lesion. In contrast, Grb2+/− mice were resistant to the development of neointima

Conclusions—Grb2 is an essential component of the signaling cascade resulting in neointima formation after arterial injury. (Arterioscler Thromb Vasc Biol. 2003;23:1788-1793.)

Key Words: vascular injury ■ signaling ■ MAPK ■ neointima ■ ischemia

Atherosclerosis often leads to ischemia or infarction of the heart, brain, kidney, and other organs and is a common cause of severe morbidity and death. Stenotic and occlusive atherosclerotic lesions are commonly treated by percutaneous transluminal coronary angioplasty or percutaneous delivery of stents. These treatment modalities tend to be highly successful in their initial application, but restenosis of arterial lesions occurs in 30% to 50% of patients within 3 to 6 months of these procedures.1,2 The molecular processes that result in restenosis are incompletely understood, but elaboration of neointima composed of matrix and smooth muscle cells (SMCs) that proliferate and migrate from the tunica media are known to contribute.3,4

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Several growth factor signaling cascades have been implicated in the pathogenesis of neointima formation. For example, platelet-derived growth factor (PDGF) is released locally by platelets and other cells in arteries after injury.5 Inhibition of PDGF signaling by infusion of blocking antibodies or by administration of a PDGF receptor tyrosine kinase inhibitor was shown to prevent neointima formation after vascular injury.6,7 Lindner and Reidy8 similarly showed that inhibition of basic fibroblast growth factor action by infusion of blocking antibodies prevented the development of neointimal hyperplasia after injury. Other growth factors and ligands are released locally in vessels after injury, including epidermal growth factor and thrombin.9,10 These ligands all bind to transmembrane receptors on the surface of SMCs that initiate intracellular signaling cascades that include the small GTPase ras. Therefore, although many ligands are released locally in response to vascular injury, their cognate receptors all tend to signal through ras.

The role of ras in the development of vascular lesions was previously examined in several animal preparations. In particular, adenoviral-mediated gene transfer into vessels of a dominant-negative mutant form of H-ras inhibited the development of stenotic lesions in rats after mechanical injury.11–14 Similar results were obtained when animals were treated with a chemical inhibitor of ras farnesyltransferase that blocks ras activity.15,16

Once activated, ras directly binds to several critical effectors, including Raf-1, B-Raf, phosphatidylinositol-3’ (PI3) kinase, and ral guanine nucleotide dissociation stimulator.17 When activated, these ras effectors trigger the Raf-MEK-extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) cascade, the PI3 kinase-PDK1-Akt cascade, and the ral cascade. In addition, ras activation results in the secondary activation of rac1 and other small GTPases that, in turn, activate the c-Jun-NH2-terminal kinase...
In this way, activation of ras leads to a variety of signaling events culminating in changes in gene expression, protein synthesis, metabolism, cytoskeletal regulation, and cell cycle progression. The activation of ras requires that a guanine nucleotide exchange factor be brought into contact with ras, which is constitutively located at the internal surface of the plasma membrane of cells. Grb2 facilitates ras activation by delivering son of sevenless protein (SOS), which is a guanine nucleotide exchange factor to ras.\(^\text{19,20}\) Grb2 binds to a polyproline region in SOS via a SH3 domain. Grb2 typically translocates from the cytosol to the plasma membrane by SH2 domain-mediated binding to phosphotyrosine motifs on the internal portion of receptor tyrosine kinases or other proteins that are located at the plasma membrane, such as Src or focal adhesion kinase.\(^\text{21,22}\)

In addition to SOS, Grb2 binds to other signaling proteins, including the Grb2-associated binder 1 (Gab1) and Grb2-associated binder 2 (Gab2) proteins, c-Abl, and dynamin.\(^\text{23–25}\) Gab1 protein is known to associate with PI3 kinase and may be involved in activation of the Akt pathway independent of ras activation.\(^\text{26}\)

Although the role of Grb2 in atherosclerosis and restenosis is not known, several studies have examined the role of Grb2 in growth factor signaling in vascular smooth muscle cells.\(^\text{21,27–29}\) Grb2 protein is present in cultured rat vascular smooth muscle cells and is recruited to the plasma membrane by cell stimulation with PDGF, angiotensin II, and mechanical stretch.\(^\text{21,27–29}\)

Grb2\(^{-/-}\) mice do not survive embryonic development because of defective endoderm differentiation and because they are unable to form the epiblast.\(^\text{30}\) However, Grb2\(^{+/+}\) mice survive embryogenesis, appear normal at birth, and are fertile. Grb2\(^{-/-}\) mice have a 40% to 50% reduction in Grb2 protein in all tissues tested to date and have a defect in T cell signaling; however, peripheral T cells function normally and animals do not develop autoimmune disease and are not sensitive to infection.\(^\text{30}\) Although ERK activation is normal in Grb2\(^{-/-}\) T cells, p38 MAPK and JNK activation are markedly reduced.\(^\text{31}\) Based on these defects in signal transduction, we hypothesized that Grb2\(^{-/-}\) mice would be resistant to neointima development in response to mechanical injury.

**Methods**

**Cell Culture**

Primary rat aortic smooth muscle cells (RAOSMCs) were obtained from Cell Application, Inc (San Diego, Calif) and grown in Rat Smooth Muscle Cell Growth Medium (Cell Application, Inc, San Diego, Calif). Primary mouse aortic smooth muscle cells (MASMCs) were isolated from Grb2\(^{-/-}\) and Grb2\(^{+/+}\) mice. Aortas were isolated, flushed with PBS, and placed in 6-well plastic culture dishes containing MEM media supplemented with 20% fetal bovine serum. Colonies of SMCs that grew from aortas were transferred to T75 tissue culture flasks. Immunohistochemical staining with an anti-\(\alpha\)-actin antibody was performed to confirm that cultured cells were MASMCs.

**Morpholino Antisense Oligos**

Morpholino antisense oligos directed against rat Grb2 (Grb2 Morpholino) and standard control morpholino oligo were obtained from Gene Tools, LLC.\(^\text{31,32}\) Subconfluent RAOSMCs were scrape loaded with Grb2 morpholino or standard control morpholino oligo as directed by the manufacturer. In brief, 20 nmol/L Grb2 Morpholino or standard control morpholino oligo was added to a culture plate and swirled briefly. Cells were scraped from the culture plate and were seeded at a concentration of 1×10\(^4\) per well in a 6-well tissue culture plate. After cells were starved in serum-free DMEM overnight to induce quiescence, the medium was replaced with growth medium or growth medium plus PDGF-BB (25 ng/mL; Calbiochem).

**Determination of Cell Number by the MTS Method**

After 96 hours, cell number was determined by use of the colorimetric method provided by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega). This assay depends on two solutions: a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine methosulfate; PMS). MTS is bioreduced by cells into a formazan product. The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in cells that are metabolically active. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture. RAOSMCs and MASMCs were suspended with trypsin and diluted. One hundred microliters of the cell suspension and 20 \(\mu\)L of assay mixture (MTS/ PMS solution) were added to each well of a 96-well plate and were incubated for 1 hour. Absorbance was measured in a microplate reader (BIO-RAD 550) at 490 nm. Cell number was also determined by direct counting under a compound microscope.

**Protein Analysis**

Cytosolic extracts of RAOSMCs and MASMCs were obtained as previously described.\(^\text{33}\) In brief, cells were washed with ice-cold PBS and lysed in protein lysis buffer (0.5% NP-40, 137 mmol/L NaCl, 50 mmol/L NaF, 5 mmol/L EDTA, 10 mmol/L Tris HCl [pH 7.5], 2 mmol/L phosphomolybdate fluoride, 25 mmol/L leupeptin, 0.2 U/mL aprotinin). In addition, aortas and carotid arteries were harvested from Grb2\(^{-/-}\) and Grb2\(^{+/+}\) mice and protein lysates were generated with a polytron in the presence of protein lysis buffer. Lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were electrophoretically transferred to nitrocellulose filters.\(^\text{34}\) Filters were blocked in Tris-buffered saline containing 1% Tween 20 and 5% nonfat dried milk and then were incubated with primary antibody. Primary antibodies used included murine monoclonal anti-Grb2 antibody and anti-ERK antibody, rabbit polyclonal anti-phospho p38 MAPK antibody, anti-phospho-JNK antibody, anti-p38 MAPK antibody, and anti-JNK antibody (Cell Signaling Technology). Filters were washed in Tris-buffered saline containing 1% Tween 20 and then were incubated with horseradish peroxidase–conjugated antirabbit or antimouse secondary antibody (Amersham). Bands were visualized by use of the ECL system (Amersham).\(^\text{35}\)

**Grb2\(^{+/+}\) Mice**

Grb2\(^{+/+}\) mice in the 129/SvJ strain were generated as previously described.\(^\text{29}\) Grb2\(^{+/+}\) mice do not survive embryonic development. Grb2\(^{-/-}\) mice were compared with Grb2\(^{+/+}\) 129/SvJ littermates in all experiments described in the article. All research involving the use of mice were performed in strict accordance with protocols approved by the Animal Studies Committee of Washington University School of Medicine.

**Carotid Injury**

The carotid arteries of 12-week-old mice were mechanically injured by use of a modification of the guide wire probe.\(^\text{34,35}\) Animals were anesthetized with intraperitoneal injection of ketamine (80 mg/kg of body weight) and xylazine (16 mg/kg body weight). The entire left carotid body was exposed by use of a modification of the guide wire probe.\(^\text{34,35}\) Animals were anesthetized with intraperitoneal injection of ketamine (80 mg/kg of body weight) and xylazine (16 mg/kg body weight). The entire left carotid artery was exposed and the proximal common carotid artery was occluded with a microvascular clamp. Another clamp was placed at the internal carotid arterial branch. A 7-0 suture was placed around the external carotid artery immediately distal to the point of
Role of Grb2 in RAOSMC Proliferation

To determine the role of Grb2 in the proliferation of SMCs, RAOSMCs were cultured to subconfluence and cytosolic lysates were obtained. Immunoblot analysis demonstrated that Grb2 protein was present at high levels in RAOSMCs (Figure 1).

To reduce Grb2 protein levels in cultured cells, an antisense oligonucleotide against rat Grb2 was used.31,32 These oligonucleotides have the riboside portion of each subunit converted to a morpholine moiety and also have phosphorodiadate intersubunit linkages.31 Morpholino oligos are reported to exhibit greater efficiency and specificity than other antisense oligos when tested in cultured cells.31,32 Subconfluent RAOSMCs were scrape loaded with the Grb2 morpholino or with a control morpholino at an identical concentration. Immunoblot analysis revealed that Grb2 protein levels were markedly reduced in Grb2 morpholino-loaded cells but not in control morpholino-loaded cells (Figure 1).

To determine whether reduced Grb2 protein levels affected the growth characteristics of SMCs, proliferation assays were performed. When stimulated with PDGF for 4 days, RAOSMCs loaded with the Grb2 morpholino proliferated at a much slower rate than control morpholino-loaded cells. Indeed, the cell number of control morpholino-loaded cells increased by 11.2 ± 1.8-fold in response to PDGF stimulation but the cell number of Grb2 morpholino-loaded cells only increased by 2.9 ± 0.6-fold.

The signaling properties of RAOSMCs loaded with the Grb2 morpholino were evaluated. Serum-starved RAOSMCs were stimulated with PDGF. After 20 minutes of stimulation, cytosolic lysates were obtained. Activation of JNK and p38 MAPK was evaluated by use of phosphospecific antibodies that determine whether the kinases are phosphorylated in the “TXY” activation loop. Immunoblot experiments revealed that JNK was activated in both unstimulated and PDGF-stimulated control morpholino-treated cells and that p38 MAPK was activated in PDGF-stimulated control morpholino-treated cells when compared with unstimulated control morpholino-treated cells. In contrast, JNK and p38 MAPK was not activated in either unstimulated or PDGF-stimulated Grb2 morpholino-treated cells (Figure 1).

Analysis of Murine Grb2<sup>−/−</sup> SMC Proliferation

To investigate the role of Grb2 in Murine SMC proliferation, Grb2<sup>−/−</sup> mice were analyzed. Grb2 protein levels in the vessels of Grb2<sup>−/−</sup> mice was evaluated by immunoblot analysis of cytosolic protein lysates derived from the aortas of Grb2<sup>−/−</sup> and Grb2<sup>+/+</sup> mice. The aortic Grb2 protein level was reduced by approximately 40% in Grb2<sup>−/−</sup> mice when compared with Grb2<sup>+/+</sup> mice. This reduction in Grb2 protein is consistent with that observed in cardiac tissue and in T cells.30,36

MASMCs were isolated from Grb2<sup>+/−</sup> and Grb2<sup>+/+</sup> mice and were stimulated with PDGF for 4 days. Cell proliferation was assessed by use of two independent techniques, including the MTS method and by direct counting. MASMCs from Grb2<sup>+/−</sup> mice exhibited a much slower proliferation rate compared with cells derived from Grb2<sup>−/−</sup> mice (Figure 2).

Role of Grb2 in the Response to Carotid Arterial Injury in Mice

Grb2<sup>−/−</sup> mice and their Grb2<sup>+/+</sup> 129/SvJ littermates were subjected to carotid injury by the epoxy resin–beaded probe.
method. Both groups of mice tolerated the procedure well with minimal mortality. Immunoblot analysis of carotid cytosolic lysates revealed that the activity of ERK, JNK, and p38 MAPK was increased in Grb2−/− mice after injury (Figure 3). In contrast, carotid p38 MAPK activity was markedly decreased in Grb2−/− mice both at baseline and after injury (Figure 3). The basal activation of ERK and JNK was attenuated in the carotid arteries of Grb2−/− mice. After injury, carotid ERK and JNK activity increased in Grb2−/− mice, but to a lesser extent than observed in Grb2+/+ mice (Figure 3).

Histological examination of carotid cross-sections 3 weeks after injury revealed a robust neointima in Grb2−/− mice (Figure 4A and 4B). In contrast, there was a 50% reduction in neointimal formation in Grb2+/+ mice (Figure 4C and 4D). Specifically the neointimal area in the injured artery was 0.038±0.009 mm² in Grb2+/+ mice and was 0.014±0.004 mm² in Grb2−/− mice (P=0.026). The neointimal-to-media ratio in the injured artery was 1.40±0.370 in Grb2+/+ mice and was only 0.47±0.150 in Grb2−/− mice (P=0.034). The contralateral carotid artery, used as a control in each animal, showed no neointimal formation.

The composition of cells in neointimal lesions was determined by immunohistochemical methods and revealed that SMCs were the major component of neointimal lesions in Grb2−/− mice (Figure 5A). In contrast, although SMCs were present in the tunica media of Grb2−/− mice, there was reduced actin staining in the neointima (Figure 5B).

We also examined the vascular signaling manifested in carotid cross-sections from Grb2−/− and Grb2+/+ mice. Using an immunohistochemical microscopy approach with antiphosphospecific antibodies, we showed that p38 MAPK activation was present in both the tunica media and in the neointima of Grb2+/+ mice (Figure 6A). In contrast, p38 MAPK activation was not detected in either the tunica media or the neointima of Grb2−/− mice (Figure 6B).

**Discussion**

Grb2 is a pivotal signaling molecule in growth factor-stimulated signal transduction. Grb2, via its interactions with SOS, promotes ras activation.19,20 Grb2 also interacts with receptor tyrosine kinases, focal adhesion kinase, Shc, Gab1, Gab2, dynamin, and other signaling molecules.21–26 As a result of these interactions, Grb2 is a molecular sensor that detects upstream activation of several receptor systems and translates that into the downstream activation of ERK, JNK, p38 MAPK, Akt, and ral GDS. Grb2 is an essential gene in invertebrate and vertebrate animals. Grb2−/− mice die at embryonic day 4 because of defective endoderm differentiation and failure to form the epiblast.29

![Figure 2](http://atvb.ahajournals.org/)

Figure 2. MASMC proliferation was analyzed by use of the MTS method. Cultured MASMCs were starved and stimulated with PDGF, and cell number was detected by the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, Wis).

![Figure 3](http://atvb.ahajournals.org/)

Figure 3. Analysis of Grb2 function in carotid arteries isolated from Grb2−/− and Grb2+/+ mice. Carotid protein lysates were analyzed by antiphospho-p38 MAPK immunoblotting (first panel, top), by anti-phospho-ERK immunoblotting (second panel) and by anti-phospho-JNK immunoblotting (third panel). Blots were re-probed with an anti-total p38 MAPK antibody (second panel), anti-total ERK antibody (fourth panel) and anti-total JNK antibody (sixth panel) to control for protein loading. Using

![Figure 4](http://atvb.ahajournals.org/)

Figure 4. A through D. Photomicrographs of cross-sections obtained 3 weeks after vascular injury (200×). Sections stained with Verhoeff’s von Giesen stain showed a prominent neointimal thickening in Grb2+/+ mice (A and B) that was markedly attenuated in Grb2−/− mice (C and D).

![Figure 5](http://atvb.ahajournals.org/)

Figure 5. Presence of SMCs in vascular lesions. Carotid cross-sections obtained from Grb2+/+ (A) and Grb2−/− mice (B) 3 weeks after injury were stained for SMC actin (200×). Notice the presence of many actin-positive cells, signified by brown staining, in the intimal lesions of Grb2−/− mice.
In this work, we have demonstrated that Grb2 protein is present in cultured vascular SMCs and that reduced Grb2 protein inhibits both rat and murine aortic smooth muscle cell proliferation and MAPK signaling (Figures 1 and 3). Two methods were used to reduce Grb2 protein levels in SMCs. A morpholino oligonucleotide “knockdown” approach was used to reduce Grb2 protein in cultured RAOSMCs. In contrast, MASMCs were directly isolated from the aortas of Grb2+/− and Grb2−/− mice. Taken together, these results show that Grb2 action is an important component of growth factor-mediated smooth muscle cell signal transduction and cell proliferation.

We next investigated the in vivo role of Grb2 in the response to vascular mechanical injury by use of Grb2−/− mice. We used an epoxy resin–beaded probe method to surgically injure the carotid artery. This mechanical injury technique reproducibly induced the development of severe neointimal lesions in wild-type mice. Immunohistochemical analysis of vascular lesions in wild-type mice revealed that they were primarily composed of SMCs. When Grb2+/− mice were subjected to carotid injury surgery, they were found to be highly resistant to neointimal lesion formation (Figures 4 and 5). Furthermore, Grb2−/− mice had decreased p38 MAPK activation in vascular SMCs after carotid injury (Figures 3 and 6). Therefore, Grb2 action is an essential component of neointimal formation in response to mechanical vascular injury, presumably because of defective SMC signaling.

It is important to note that vascular lesions that develop in response to mechanical injury may be different from those that develop as a consequence of the chemical injury that may occur with abnormal lipid metabolism. Signaling proteins that promote neointima formation in response to vascular mechanical injury may have a different effect on the development of atherosclerotic lesions in animals with lipid abnormalities. Therefore, it will be interesting to determine whether Grb2−/− mice are resistant to the development of atherosclerotic lesions that occur in Apo protein E−/− or LDL receptor−/− mice.

Grb2 represents a target of drug discovery for the treatment and prevention of vascular restenosis. This is particularly true because a reduction of only one copy of the Grb2 gene almost completely inhibited the development of neointimal lesions in response to vascular mechanical injury. In particular, Grb2 may be a useful target for antisense oligonucleotide therapy in vivo. Additionally, the development of small molecule inhibitors of the Grb2-SOS interaction may be beneficial for the treatment of patients.

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