Gas6 Anti-apoptotic Signaling Requires NF-κB Activation*

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Francesca Demarchi‡§, Roberto Verardo‡, Brian Varnum‖, Claudio Brancolini‖, and Claudio Schneider‡§**

From the ‡Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie AREA Science Park, Padriciano 99, Trieste 34012, Italy, the §Dipartimento di Scienze e Tecnologie Biomediche, Universita’ degli Studi di Udine, piazzale Kolbe 4, Udine 33100, Italy, and ‖Amgen Inc., Thousand Oaks, California 91320

The growth arrest-specific 6 gene product Gas6 is a growth and survival factor related to protein S. Gas6 is the ligand of Axl receptor tyrosine kinase; upon binding to its receptor Gas6 activates the phosphatidylinositol 3-OH kinase (PI3K) and its downstream targets S6K and Akt. Gas6 anti-apoptotic signaling was previously shown to require functional PI3K and Akt and to involve Bad phosphorylation in serum-starved NIH 3T3 cells. Here we demonstrate that Gas6 induces a rapid and transient increase in nuclear NF-κB binding activity coupled to transcription activation from NF-κB-responsive promoters and increase in Bcl-xL protein level. Gas6 survival function is impaired in cells lacking p65/RelA and in NIH 3T3 cells transfected with a dominant negative IκB, indicating that NF-κB activation plays a central role in promoting survival in this system. Moreover, NF-κB activation can be blocked by a dominant negative Akt and by wortmannin, an inhibitor of PI3K, thus suggesting that NF-κB activation is a downstream event with respect to PI3K and Akt, as already described for other growth factors. In addition, we show that glycogen synthase kinase 3, which is phosphorylated in response to other growth factors. In addition, we show that glycogen synthase kinase 3 in Gas6 anti-apoptotic signaling and unveil a possible link between these survival pathways.

The growth arrest-specific 6 gene product (Gas6) is a secreted protein related to the anti-coagulant protein S. Gas6 binds, with different affinities, to the receptors of the mammalian Axl protein-tyrosine kinase family: Axl (also named Ark, Ufo, Tyro7), Rse (also named Sky, Bt, Tif, Dtk, Tyro3), and Mer (Eyk, Nyk, Tyro12). Inactivation of the Gas6 gene has recently been shown to prevent venous and arterial thrombosis in mice and protect against fatal collagen/epinephrine-induced thrombembolism (1). This knock-out phenotype has unveiled one of the biological functions of Gas6; however, published data of the past several years argue for a multiplicity of functions of GAS6/...
via the ubiquitin-proteasome pathway. The released NF-κB dimer can then translocate to the nucleus, where it directly binds to its cognate DNA sequence to activate gene transcription.

NF-κB is a regulator of inflammation and immune response as well as of cellular proliferation and apoptosis (23). Although NF-κB has been shown to be pro-apoptotic in certain experimental settings, a number of data argues for a role of NF-κB as regulator of survival. RelA (p65) knock-out in mice is embryonically lethal as a result of extensive liver apoptosis. In addition, cells derived from these mice show enhanced sensitivity to TNF-induced apoptosis (24). NF-κB is required for protection from apoptosis occurring after growth factors withdrawal in various systems, including fibroblasts (16), hematopoietic cells (19), hepatoma cells (25), Chinese hamster ovary (CHO) (26), and PC12 cells, (27). A number of anti-apoptotic proteins encoded by NF-κB-inducible genes have recently been identified, namely Bcl-xL (28, 29), A1 (30, 31), and TNF receptor-associated proteins 1 and 2 (32).

RelA binds to its cognate DNA sequence to activate gene transcription. The substrates were obtained using the Dual Luciferase Buffer (Promega). Extracts were collected and cleared by centrifugation at high speed. The substrates were obtained using the Dual Luciferase Reporter assay system (Promega), and relative light units (firefly/Renilla) were measured using a luminometer (Turner Design).

Western Blot—Western blot analysis was performed by preparing whole cell extracts in 2× SDS sample buffer containing 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin on ice. 20 μg β-glycerophosphate, 10 μM sodium orthovanadate was added to the extracts to be analyzed with anti-phospho-GSK3 antibodies. Western blotting antibodies were purchased from the following companies: IxB (New England Biolabs, Inc., Beverly, MA), NF-κB p50/105 (Santa Cruz Biotechnology, Inc.), Bcl-x (Transduction Laboratories, Lexington, KY), GSK3 (BIOSOURCE International Inc.), Phospho GSK3 (Cell Signaling), and actin (Sigma). After incubation with primary antibodies, blots were incubated with horseradish peroxidase secondary antibodies (Sigma) and visualized using ECL chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Cell Culture and Reagents

Cell culture media and reagents were obtained from the following sources: Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 μg/ml). Medium was replaced with 0.5% FCS containing serum for 48 h to induce growth arrest by serum starvation. Wortmannin, sodium fluoride, and sodium orthovanadate were from Sigma Chemical Co., St. Louis, MO. Basic fibroblast growth factor (bFGF) was kindly supplied by Dr. C. Grassi (Farmitalia, Milano). Epidermal growth factor (EGF) was kindly provided by A. Ullrich. Recombinant Gas6 was supplied by Amgen Inc.

Transfection and Luciferase Reporter Assay—NIH 3T3 cells at 60–80% confluency were transiently transfected using LipofectAMINE Plus reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. IxBΔN in pCMV4 expression vector was kindly provided by Dr. Shao-Cong Sun and is described in a previous study (37), pCMV6 plasmids containing HA-akt K– and AKT K– (K179M) were kindly provided by Dr. A. Bellacosa. Basic Tkiuc containing the herpes simplex virus thymidine kinase (TK) promoter in front of luciferase and mPRDII TKluc, with two NF-κB binding sites in front of the TK promoter, were a kind gift of Dr. D. Maniolo and have been described previously (38). Bcl-xL promoter and bcl-x promoter triple mutant linked to luciferase expression vector were a kind gift of Dr. Perez Polo and are described in a previous study (29). PRL-null Renilla luciferase expression vector was from Promega (Madison, WI). Briefly, 1–2 μg of plasmid DNA were diluted in Opti-MEM (Life Technologies, Inc.) and mixed with LipofectAMINE and Plus reagent. Complexes were allowed to form for 15 min prior to addition to the cells. Meanwhile the culture medium was replaced with Opti-MEM. The medium was replaced with 0.5% FCS containing medium 5 h after transfection to induce growth arrest. 48 h later the medium was replaced with serum-free medium with or without Gas6, and the cells were lysed 5 h later in Passive Lysis Buffer (Promega). Extracts were collected and cleared by centrifugation at high speed. The substrates were obtained using the Dual Luciferase Reporter assay system (Promega), and relative light units (firefly/Renilla) were measured using a luminometer (Turner Design).

RESULTS

Gas6 Treatment Is Coupled to a Decrease in IxBα Protein Level and to an Increase in Nuclear NF-κB Binding Activity in NIH 3T3 Mouse Fibroblasts—Gas6 anti-apoptotic activity was previously shown to absolutely require the phosphatidylinositol-3-OH kinase (PI3K) (14) and its substrate AKT/PKB (15) in serum-starved NIH 3T3 mouse fibroblasts. AKT has been shown to drive transcription activation of NF-κB both indirectly through IKK and subsequent IκB degradation and directly through IκB phosphorylation (16, 17) or directly by p65/RelA subunit phosphorylation (42, 43).

As a first approach to verify whether Gas6 could lead to NF-κB induction, we analyzed the effect of Gas6 treatment on the protein level of the nuclear NF-κB inhibitor IκB-α. NIH 3T3 cells were grown for 48 h in 0.5% containing medium to achieve quiescence. Thereafter the medium was replaced with serum-free containing medium in the presence or absence of Gas6 and lysesates prepared at different time points (5, 15, 30, 60 min). Some plates were replaced with 20% FCS containing medium as control for IκBα down-regulation. The blot shown in Fig. 1A demonstrates that both Gas6 and 20% FCS treatment are coupled to a transient and rapid decrease in IκBα. Actin was used as loading control.

To analyze the effect of Gas6 on nuclear NF-κB binding activity, gel retardation assays were performed with a 32P-labeled oligonucleotide containing a NF-κB consensus as already described (39). NIH 3T3 cells were serum-starved (48 h in 0.5% FCS) and then shifted to 20% FCS or to serum-free medium with or without Gas6, and nuclear extracts were prepared 60 min later. Extracts were prepared also from cells left in 0.5% FCS as negative control. Fig. 1B shows the retarded
bands. Shifting the cells to serum-free induces a slight increase in binding activity (lane 2) as compared with growth-arrested control cells (lane 1). Gas6 (lane 4) induces a clear increase in the complex binding to the κB-specific oligonucleotide, just as 20% serum (lane 3), used as positive control, because it was previously shown to induce NF-κB (40). The specificity of the retarded complex was assessed by competition experiments with cold specific and unspecific oligonucleotides as already described (39) (data not shown).

NF-κB Is Required for Gas6-mediated Protection from Apoptosis—Because we have observed that Gas6 treatment is coupled to the induction of nuclear NF-κB binding activity, we investigated the effect of other growth factors in our experimental setting. Serum-starved cells were shifted to serum-free medium, and FGF, EGF, or Gas6 were added to the cells. Control cells were shifted to serum-free medium without growth factors. Treated cells were either used to prepare nuclear extracts 30 min later, or to measure viability 20 h later. The graph reported in Fig. 2A indicates the increase in cell viability with respect to the control cells grown in serum-free medium. Both Gas6 and FGF, but not EGF, efficiently protect serum-starved NIH 3T3 fibroblasts from apoptosis, as already reported (8). Interestingly, protection from apoptosis correlates with a clear increase of NF-κB binding activity, as shown in Fig. 2B. The κB binding activity induced by FGF and Gas6 are comparable, whereas it is significantly lower in extracts from EGF-treated cells.

To test the importance of NF-κB induction in Gas6-mediated protection from apoptosis, we challenged growth-arrested mouse fibroblasts lacking either p50 (41) or p65 (24) subunits of NF-κB with serum-free medium in the presence or absence Gas6. As reported in the graph of Fig. 2C, p50−/− cells can be rescued by Gas6, as for wild type NIH 3T3 cells, whereas p65−/− cells cannot. This result demonstrates that Gas6-mediated protection from apoptosis induced upon serum withdrawal requires a functional cRel/p65, whereas p50 is dispensable.

To further investigate the relevance of NF-κB induction for Gas6 survival properties, we analyzed the effect of a dominant negative IκBα (IκBα ΔN) on Gas6-mediated protection from apoptosis. NIH 3T3 were cotransfected with dominant negative IκBα or with an empty vector as transfection control. 6 h later, the cells were serum-starved for 48 h to achieve growth arrest. Thereafter, the cells were incubated in serum-free medium in the presence or absence of Gas6 or in 0.5% containing medium for additional 20 h. Afterward, the cells were trypsinized and counted. The graph indicates the increase in cell viability of Gas6-treated cells with respect to control cells incubated in serum-free medium without growth factors. A, serum-starved cells (48 h in 0.5% serum containing medium) were incubated with the FGF, EGF, and Gas6 in serum-free medium for 20 h; then the cells were trypsinized and counted. The graph indicates the increase in viability with respect to the control cells incubated in serum-free medium without growth factors. B, serum-starved cells were incubated with FGF, EGF, and Gas6 in serum-free medium for 1 h, and nuclear extracts were prepared and analyzed in gel retardation assays with a NF-κB-specific oligonucleotide. C, effect of Gas6 on cell viability of p50−/− and p65−/− murine fibroblasts. NIH 3T3 cells were used as control. The graph indicates the increase in cell viability of Gas6-treated cells with respect to control cells incubated in serum-free medium. D, effect of a dominant negative IκBα on Gas6-mediated protection from apoptosis. NIH 3T3 were cotransfected with dominant negative IκBα or with an empty vector as transfection control. 6 h later, the cells were serum-starved for 48 h to achieve growth arrest. Thereafter, the cells were incubated in serum-free medium in the presence or absence of Gas6 or in 0.5% containing medium for additional 20 h. Afterward, the cells were trypsinized and counted. The graph shows the average of the number of recovered cells in three independent experiments.
in phosphate-buffered saline and incubated in serum-free plus or minus Gas6 or again with 0.5% serum containing medium for additional 20 h. At this point the cells were trypsinized and counted. The graph of Fig. 2D reports the average of the results obtained in three independent experiments and clearly demonstrates that blocking NF-κB activation by a dominant negative IκB impairs Gas6 survival activity.

NF-κB Binding Activity Induction by Gas6 Is Rapid and Transient and Contains p50 and p65 Subunits—To analyze the kinetics of NF-κB binding activity induction, we prepared nuclear extracts at different time points after shifting serum-starved NIH 3T3 cells to serum-free medium containing Gas6 and luciferase activity induction, we prepared nuclear extracts either with p50-, p65-, or cRel-specific antibodies against the p50 (lane 2), p65 (lane 3), or cRel (lane 4) and subsequently used for gel retardation assay. The control assay, preincubated without antibodies, is shown in lane 1.

To identify the polypeptides present in the shifted complex we preincubated the nuclear extracts either with p50-, p65-, or cRel-specific antibodies before performing the gel retardation assay. As shown in Fig. 3B, both p50 and p65 antibodies can partially super-shift the retarded complex, whereas cRel-specific antibody cannot. These data indicate that Gas6-induced complex contains both p50 and p65 subunits of NF-κB but not cRel.

Gas6 Induces NF-κB-dependent Transcription Activation—The effect of Gas6 on NF-κB-dependent transcription was studied by analyzing its effect both on an artificial promoter containing two NF-κB binding sites and on the bcl-x promoter. NIH 3T3 cells that were transfected with luciferase expression plasmids driven by the herpes simplex virus thymidine kinase (TK) promoter or the same promoter containing two NF-κB binding sites (38). A Renilla expression vector was included in each transfection experiment for normalization. 6 h after transfection the cells were rinsed and the medium was replaced with 0.5% FCS containing medium to induce growth arrest. 48 h later the medium was changed with serum-free medium with or without Gas6 for an additional 5 h. Afterward, relative luciferase activity (firefly/Renilla) was measured with a luminometer.

Fig. 4A shows the increase of relative luciferase (LUC) activity occurring for each transfected expression vector using extracts from Gas6-treated cells as compared with control cells. These results indicate that Gas6 can specifically increase the transcription rate from a promoter containing binding sites for NF-κB. Similar results were obtained by transfecting a LUC expression vector driven by the bcl-x promoter or a bcl-x promoter in which the three putative NF-κB binding sites have been mutated (29). As shown in Fig. 4B, Gas6 can positively affect the bcl-x promoter but not the same promoter lacking NF-κB binding sites. Altogether these data argue that Gas6 can activate NF-κB-dependent transcription.

To verify the biological relevance of the increase in bcl-x promoter activity in response to Gas6, we analyzed its effect on Bcl-xL protein level in a time-course experiment. Cell lysates from serum-starved NIH 3T3 cells incubated for different times with Gas6 were analyzed in a Western blot decorated with an antibody specific for Bcl-xL; actin was used as loading control. As shown in Fig. 4C, Gas6 treatment is coupled to an increase in Bcl-xL protein level, suggesting that one of the mechanisms by which Gas6 can protect from apoptosis is by up-regulating...
the anti-apoptotic protein Bcl-x. To further assess the relevance of NF-κB activation by Gas6 in Bcl-x up-regulation, the same type of analysis was performed in serum-starved p65⁻/⁻ and p50⁻/⁻ cells. Fig. 5A shows that Bcl-xL promoter activity appears to be susceptible to Gas6 induction in p50⁻/⁻ cells, whereas it is unaffected in p65⁻/⁻ cells. The blot of Fig. 5B shows that Gas6 induces Bcl-xL protein increase in p50⁻/⁻ cells, but not in p65⁻/⁻ cells, suggesting that a functional p65 is required for the noticed effect of Gas6.

**NF-κB Activation by Gas6 Involves the PI3K and AKT Pathways**—Previous studies have demonstrated the absolute requirement both of PI3K and AKT for Gas6 survival property (14, 15). To verify whether NF-κB activation was a downstream event in these pathways we analyzed the effect of a dominant negative Akt on Bcl-xL promoter activation in response to Gas6 in serum-starved NIH 3T3. As shown in Fig. 6A, the increase in the Bcl-xL promoter activity upon Gas6 addition can be blocked by co-transfecting an expression plasmid encoding for a dominant negative, kinase dead Akt. As a first approach to investigate whether this in vitro phosphorylation could have any biological significance, we investigated whether the endogenous p105 and GSK3 do interact in living cells. To this end we probed a p50/p105 immunoprecipitation product with a GSK3-specific antibody. A total lysate was incubated with wortmannin and used it in an in vitro kinase assay with recombinant GSK3B; a mock immunoprecipitation product was used as negative control. A radioactive band of 105-kDa apparent molecular mass specifically appears after incubation of the p50/p105 immunoprecipitation product with Gas6, as shown in Fig. 7B.

To address the question whether Gas6 signaling affects the association between p105 and GSK3, we analyzed the p105 protein level in cells treated with Gas6 and in control, untreated cells. As shown in Fig. 7D, the level of p105 significantly decreases in response to Gas6. This result indicates that the association between p105 and GSK3 may be regulated indirectly by Gas6, through a reduction in p105 protein level.

**DISCUSSION**

The data presented in this manuscript demonstrate the requirement of transcription factor NF-κB activation in Gas6/Axl signaling and protection from apoptosis. We have used NIH 3T3 mouse fibroblasts as a model system to investigate Gas6/Axl function in the control of apoptosis, as already characterized (8, 14, 15). Previous studies have shown that Axl receptor
activation by Gas6 switches the PI3K/AKT pathways on and that both of them are absolutely required for protection from apoptosis in this system (14, 15). In addition, Gas6 treatment is coupled to phosphorylation and inactivation of the pro-apoptotic protein Bad (15). Here we show that NF-κB induction and transcription activation require functional PI3K and Akt kinase. Therefore, on the basis of published data and the results reported here we can suggest that the Gas6 survival pathway involves consecutive activation of Axl, PI3K, and Akt, ultimately leading to an increase in nuclear NF-κB binding activity and subsequent induction of NF-κB-responsive anti-apoptotic genes like bcl-xL.

Protection from apoptosis through the PI3K and subsequent NF-κB activation has been shown to occur in response to platelet-derived growth factor (PDGF), tumor necrosis factor (TNF), and interferon α/β (16–18). In the case of PDGF and TNF the Akt kinase can directly phosphorylate and activate the IKKα kinase with subsequent IκB phosphorylation and degradation. On the other hand, other reports argue for a direct action of Akt on the p65 subunit of NF-κB leading to transcription activation (42). In the present study we observed a transient decrease in IκBα, suggesting that also in our experimental settings the classical pathway of NF-κB activation could be switched on. Namely: activation of a IκB kinase by Akt, phosphorylation and ubiquitination-dependent degradation of IκBα, and subsequent nuclear translocation of the active transcription factor. However, we cannot exclude a direct effect of Akt on p65; indeed, this polypeptide plays a crucial role in our system. We have shown that p65 is one of the major components of the induced complex by means of super-shift analysis and that p65−/− cells are not responsive to the survival effect of Gas6.

One of the means by which NF-κB displays its pro-survival function is through the up-regulation of genes encoding for anti-apoptotic proteins (23). These proteins include Bcl-xL, TRAF1, TRAF2, c-IAP1, c-IAP2, numerous cytokines and growth factors, as well as adhesion molecules (23). Bcl-xL protein, like Bcl-2, is known to dimerize with Bax and Bad proteins, and it has been shown that the balance between the expression of these apoptosis-protecting and apoptosis-inducing proteins is critical for cell survival or death (44). Previous studies have shown that one of the effects of Gas6/Axl anti-apoptotic signaling is Bad phosphorylation (15). Here we investigated the effect of Gas6 on Bcl-xL, because the promoter of its gene has recently been shown to contain three binding sites for NF-κB and to be responsive to NF-κB activation (28, 29). We have shown that induction of NF-κB binding activities is coupled both to bcl-xL promoter activation and increase in Bcl-xL protein levels. These findings further support the relevance of the role played by Gas6 in modulating cell survival by targeting different members of the Bcl 2 family. This effect occurs as an early response through phosphorylation of Bad via Akt, followed by increased Bcl-xL expression level through NF-κB induction. In our study we have analyzed the effect of Gas6-mediated NF-κB activation on Bcl-xL; it is likely that also other NF-κB-responsive genes might be involved.

What is the physiological significance of Axl/Gas6 anti-apoptotic signaling? In the testis, one of the body districts where both Gas6 and Axl are expressed, Gas6/Axl signaling has been shown to exert anti-apoptotic functions. Mice lacking all the three receptors are indeed sterile because of progressive death of differentiating germ cells (4). Interestingly, bcl-xL is highly expressed in human spermatagonia (45) and its overexpression in mouse male germinal cells make them more resistant to apoptotic-inducing conditions (46).

Another system where Axl is highly expressed both during development and in the adult is the central nervous system (47, 48). In this system NF-κB could also play a survival function (49, 50). In addition, gene targeting has revealed that bcl-xL is required for neuronal survival during neuronal development and for post-natal CNS neurons (51). A cross-talk between GSK3β and NF-κB molecular pathways has been recently suggested because cells lacking GSK3β are defective in NF-κB signaling (36). Moreover, both GSK3β and RelA gene disruption result in embryonic lethality caused by severe liver degeneration (24, 36).

We have observed that Gas6 treatment is coupled to GSK3β phosphorylation in serum-starved NIH 3T3, as already described for the murine C57MG cells (11). This coupling could underlie an involvement of GSK3 in Gas6 anti-apoptotic signaling, as suggested for FGF. The herein reported physical association of endogenous p105 and GSK3β in living cells and the in vitro phosphorylation of p105 by GSK3 could be one of the molecular basis of a cross-talk between NF-κB and GSK3β. We speculate that GSK3 could be involved in regulating p105 protein stability. When GSK3 is phosphorylated and inactivated upon treatment with Gas6 or other growth factors, p105 could become a target of inducible kinases like ikB kinase, which target it to degradation, thus leading to NF-κB activation. Indeed we have observed that Gas6 treatment is coupled to a decrease in p105 protein level, as already reported for other NF-κB inducers. Further studies are required for a full understanding of the role played by GSK3 in Gas6-Axl signaling and NF-κB activation.

Besides its reported physiological roles in the hematopoietic and reproductive systems, Gas6/Axl signaling has also been suggested to play a role in some disorders, including gomulonephritis (52), osteoclastic bone re-absorption (2), rheumatoid arthritis (13), and certain metastatic tumors (7). A molecular
dissection of the pathways involved therefore represents an important step in the development of new tools for therapeutic intervention.

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