JOURNAL OF CELLULAR PHYSIOLOGY 204:36–44 (2005)

ORIGINAL ARTICLES

Gas6 Induces Proliferation in Prostate Carcinoma Cell Lines Expressing the Axl Receptor

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Axl is a tyrosine kinase receptor and although it is expressed in malignancy such as leukemia, colon cancer, melanoma, endometrial, prostate and thyroid cancers, its role has not been completely elucidated yet and appears to be complex. The ligand of Axl, Gas6, is a 75 KDa multimodular protein with an N-terminal gamma-carboxy-glutamic acid that is essential for binding. Gas6 has a mitogenic effect on several normal cell lines. The receptor Axl is expressed in primary prostate carcinoma and in prostate cancer cell lines as such as PC-3 and DU 145. We demonstrated a mitogenic activity determined by Gas6/Axl interaction in these undifferentiated metastatic human prostatic cancer cell lines. This effect is proportional to Axl expression, not due to inhibition of apoptosis, and induces AKT and MAPK phosphorylation. However, only MEK phosphorylation seems to be essential for growth signaling. Our results suggest that Axl overexpression and activation by Gas6 could be involved in progression of prostate neoplastic disease. J. Cell. Physiol. 204: 36–44, 2005. © 2004 Wiley-Liss, Inc.

Axl, also called Ark, belongs to a transmembrane receptor tyrosine kinases (RTKs) family (Janssen et al., 1991; Rescigno et al., 1991), which includes the Rse/Tyro3 (Lai and Lemke, 1991; Lai et al., 1994) and Mer receptors (Graham et al., 1994). This family is characterized by an unique extracellular composition of immunoglobulin-like and fibronectin III-like domains (O'Bryan et al., 1991). This architecture is also found in adhesion molecules of the cadherin and immunoglobulin superfamily and in receptor protein tyrosine phosphatases (Edelman and Crossin, 1991; Fischer et al., 1991). These structural findings suggest that Axl family members may be involved in both cell adhesion and intracellular signaling (O'Bryan et al., 1991; Bellosta et al., 1995; Goruppi et al., 1996, 1997, 1999, 2001; Avanzi et al., 1998). Axl is ubiquitously expressed in a variety of cells including normal hematopoietic cells (Neubauer et al., 1994), endothelial cells, and other mesenchimal cells (Nakano et al., 1996; Loeser et al., 1997; Avanzi et al., 1998). Many studies showed that Axl is overexpressed in malignancies such as human myeloid leukemia (Janssen et al., 1991; O'Bryan et al., 1991; Neubauer et al., 1994), colon cancer (Craven et al., 1995), hepatocellular carcinoma cell lines (Tsou et al., 1998), melanoma cell lines and in aggressive melanoma (Quong et al., 1994; Bittner et al., 2000), in primary breast cancer (Berclaz et al., 2001), and in thyroid carcinomas (Ito et al., 1999, 2002). In spite of the observations reported above, the role of Axl in normal cells and in malignancy has not been completely elucidated and appears to be complex. All members of the Axl RTKs family can transform NIH-3T3 cells, in particular Axl transforming activity is weak and depends on the host cell type and on its level of expression (Burchert et al., 1998).

The ligand of Axl, Gas6 (growth arrest specific gene 6), is a 75 KDa multimodular protein with an N-terminal γ-carboxy-glutamic domain, four EGF-like sequences, and a double globular C-terminal domain (Manfioletti et al., 1993; Varnum et al., 1995). Gas6 was originally isolated as a growth arrest specific gene, since its expression is up-regulated during the G_0–G_1 phase of the cell cycle (Schneider et al., 1988), however, its potential effect as a cell cycle regulator was never demonstrated. Vitamin K-dependent γ-carboxylation of the glutamic acid residues of Gas6 is essential for its activity and receptor binding (Nakano et al., 1997). Binding of Gas6 to Axl causes receptor dimerization and autophosphorylation with the consequent activation of tyrosine residues in its kinase domain and binding of the signaling adaptors to its multiple substrate docking sites (Braunger et al., 1997).

Gas6 has mitogenic effect on in vitro cultured NIH-3T3 fibroblasts (Goruppi et al., 1996, 1997), Schwann cells (Li et al., 1996), chondrocytes (Loeser et al., 1997), vascular smooth muscle cells (VSMCs) (Nakano et al., 1995), and C57MG human mammary carcinoma cells (Goruppi et al., 2001). However, in all those systems Gas6 appears to be a weak mitogen and seems to have preferentially a role in modulating cellular responses to other factors (Nakano et al., 1995; Gallicchio et al., 2004).

Another relevant effect of Gas6/Axl activation is to mediate a survival signaling. Studies have evidenced that Gas6 has an anti-apoptotic effect on NIH-3T3 fibroblasts (Bellosta et al., 1997; Goruppi et al., 1997, 1999), human hematopoietic stromal cells (Avanzi et al., 1997), gonadotropin-releasing hormone (GnRH)

Contract grant sponsor: Università del Piemonte Orientale.

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Received 21 September 2004; Accepted 8 October 2004

DOI: 10.1002/jcp.20265

neurons (Allen et al., 1999), endothelial cells (Hasanbasic et al., 2004), and oligodendrocytes (Shankar et al., 2003). Moreover the phenotype of mice lacking the Axl family of receptors display substantial apoptosis in the male germ line (Lu et al., 1999).

The analysis of signal transduction suggests that the mitogenic and anti-apoptotic effects of Gas6 occur through different pathways depending on cell type. In NIH-3T3 cells, Gas6 determines phosphorylation of phosphatidylinositol 3-OH kinase (PI3K) and consequent activation of the serine-threonine kinase AKT/protein kinase B (AKT/PKB) (Goruppi et al., 1997). This effect was demonstrated to be dependent also on Ras activation (Goruppi et al., 1999). However, Gas6 activation of AKT/PKB determines NF-κB nuclear translocation (Demarchi et al., 2001) and this effect is independent from Ras, and involves Rac and Rho and p38 MAPK downstream, without MEK activation (Goruppi et al., 1999). In C57MG human mammary carcinoma cells, the presence of Gas6 in the medium induces growth after confluence and similarly causes cell cycle re-entry of density-inhibited cells, interestingly this signaling is PI3K dependent and requires activation of Ras (Goruppi et al., 2001). In addition Gas6 protects GnRH neurons from programmed cell death through both ERK and PI3K/AKT pathways activation (Allen et al., 1999). This effect has also been described in cultured human oligodendrocytes where PI3K is activated but the signal transduction does not involve ERK (Shankar et al., 2003). Thus signaling downstream Axl activation is complex and produces different effects depending on cellular background.

Prostate cancer continues to be one of the biggest health problems for the aging male (Jemal et al., 2003). The metastatic malignant disease is the single most important cause of increasing morbidity and subsequent mortality of prostate cancer. The transformation of a normal cell into a tumor cell and the subsequent formation of metastasis involves a cascade of multiple events: in prostate cancer one of them is the progression to hormone independent status that leaves physician with very few treatment options. In a significant subgroup of tumors, changes in androgen receptor (AR) pathways are involved in the progression of the disease, however, other pathways, independent of AR have been suggested (Nelson et al., 2003). The expression of members of Axl family receptors in the prostate carcinoma xenograft CWR22 was firstly demonstrated by Robinson et al. (1996). Afterwards other authors demonstrated that Axl mRNA was present in normal and neoplastic prostate cancer and was overexpressed in invasive prostate cancer cell lines (Jacob et al., 1999). Since overexpression of Axl has been demonstrated to be transforming in some cell types, we wanted to investigate if Gas6, the natural ligand of Axl, could play any role, either on cell survival or proliferation, in an in vitro prostate tumor model. We choose two "classical" prostate cell lines to test the proliferative activity of Gas6. We demonstrated that both PC-3 and DU 145 cell lines are stimulated to proliferate by Gas6, however, this proliferative response strictly correlates with the expression of the Axl receptor, being higher in DU 145 cells. We also showed that the mechanism of induction of cell proliferation is not mediated by inhibition of apoptosis. The analysis of signal transduction on DU 145 cells demonstrated that PI3K/AKT and MEK pathways are activated and MEK phosphorylation seems to be essential for growth signaling.

MATERIALS AND METHODS

Cell culture and reagents

DU 145 and PC-3 cell lines were obtained from Interlab Cell Line Collection (ICLC) of the National Institute for Cancer Research (Genova, St. Louis, MO). COS-7 cell line was obtained by L. Pegoraro (University of Torino, Italy). Cell lines were grown in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 2 mM L-Glutamine (Sigma), 100 UI/ml Penicillin (Pharmacia & Upjohn Italia, Milano, Italy), 100 μg/ml Streptomycin (Bristol-Myers Squibb, Sermoneta, Italy), and with 10% fetal bovine serum (Euroclone, Wetherby, United Kingdom). Recombinant Human Gas6 and Axl-X (a soluble form of Axl receptor) were kindly donated by Dr. Brian Varnum (Amgen, Inc.). Wortmannin (a specific inhibitor of PI3K) and U0126 (a specific inhibitor of MEK1 and MEK2) were obtained from Sigma.

Cloning human Gas6 in expression vector

Human Gas6 cDNA (generous gift of Dr. Claudio Schneider, Dipartimento di Scienze e Tecnologie Biomediche, University of Udine, Italy) was cloned in pcDNA3.1(−) expression vector (Invitrogen, Carlsbad, CA) using the NOT I and HIND III sites. Cell line transfection: 10 μg of recombinant hGas6pcDNA3.1 plasmid or pcDNA3.1 (Mock) plasmid were transfected in DU 145 cell line using the DOTAP liposomal reagent (Roche, Basel, Switzerland). Transfected cells (DU 145 Gas6 and DU 145 Mock) were grown in the presence of geneticin (MP Biomedicals, Irvine, CA) at concentration of 500 μg/ml for 2 months. Cells were then reseeded and cultured in RPMI with 10% FBS, 500 μg/ml of geneticin, and 4 μM of menadione sodium bisulfite (Sigma) to permit the γ-carboxylation of glutamic acid residues of Gas6 (Bellosta et al., 1997).

Immunofluorescence staining

Cells were seeded on cover slips in 3 cm Petri dish in RPMI supplemented with 10% FBS. The day after, cover slips were washed in phosphate buffered saline (PBS) and cells fixed and permeabilized with ice cold methanol for 10 min at −20°C. A specific binding was blocked by incubation with Universal Blocking Reagent (Bio Genex, San Ramon, CA). The cover slips were then incubated at room temperature with primary antibody: anti Gas6 monoclonal IgM antibody clone 3C12 (previously obtained in our laboratory, Avanzi et al., 1998) for 60 min, washed with PBS and stained at room temperature with FITC-conjugated goat anti mouse μ chain IgG antibody (Sigma), respectively, for 60 min. Cover slips were then washed with PBS, mounted, and observed with a fluorescence light microscope (Olympus Italia, Segrate, Italy).

Cell proliferation assay

4×10^3 cells/well were washed and seeded in 96-wells plates and starved in serum-free RPMI medium for 24 h. Human purified Gas6 was added at different concentrations (500 ng/ml and 1 μg/ml) and Axl-X was added at 24 and 48 h after seeding. 1 μCi/well of Methyl-^{3}H thymidine (Amersham Biosciences, Piscataway, NJ) was added for 12 h. Cells were washed with PBS and harvested in a cell harvester (Skatron, Lier, Norway). Filter membrane incorporated radioactivity was measured in a scintillation beta counter (Tri Carb Liquid Scintillation Counter, Perkin Elmer, Boston, MA) in the presence of scintillation cocktail (Insta-Gel plus, Perkin Elmer). The results obtained in counts per minute were then calculated as average percent variation with respect to negative controls ± SD. Every assay was performed in 12 duplicates and repeated three times. Unstimulated starved cells were used as negative controls. In some experiments we used DU 145 wt, DU 145 Mock and DU 145 Gas6 cells (see below). Cells were starved in serum-free RPMI medium with 5 μg/ml of insulin (Eli Lilly & Co, Indianapolis, IN), 5 μg/ml of transferrin (Sigma), and 4 μM of menadione sodium bisulfite for 24 h (Sigma). Gas6 was added after 24 and 48 h at a concentration of 200 ng/ml.

For treatment with inhibitors: wortmannin (10 nM, 100 nM, and 1 μM) and U0126 (100 nM, 1 μM, and 10 μM) were added 30 min before incubation with Gas6. ^{3}HTdR assay was then performed as described. The possible toxicity of inhibitors was tested by adding wortmannin (1 μM) and U0126 (10 μM) to DU

145 wt cells after 24 h of serum starvation. After 24 h, cells were washed twice with PBS and resuspended in RPMI containing 1% or 5% or 10% FBS. Incorporation of ^{3}HTdR was performed for 12 h. Every assay was performed in 12 wells and repeated three times. Results were expressed as mean of percent variation of CPM obtained in cells stimulated with Gas6 versus Gas6 stimulated cells plus inhibitors. For toxicity experiment we evaluated the percentage of variation of CPM obtained in cells treated with inhibitors versus cells untreated.

Immunoprecipitation and Western blotting

Cells were seeded in 10 cm plates and grown to sub confluence in RPMI medium containing 10% FBS. After starvation in serum free for 24 h, cells were stimulated with 200 ng/ml Gas6 for 5, 15, and 30 min. After washing in cold PBS, cell extract were prepared using 1% Triton buffer (150 mM NaCl, 50 mM Hepes, 1 mM EDTA, 1% Triton, pH 7.5) containing proteases and phosphatases inhibitors (10 μg/ml aprotinin, 100 μg/ml PMSF, 100 mM sodium orthovanadate, 50 mM sodium fluoride; Sigma). Cell extracts were incubated with a polyclonal antiserum raised against the mouse homolog Ark (Bellosta et al., 1995) and protein A-sepharose (Sigma) at 4°C for 3 h. Immunoprecipitated were run on a SDS–PAGE and immunoblotting was performed using anti-phosphotyrosine antibody (clone 4G10, Upstate, Charlottesville, VA) or anti Ark antisera (Bellosta et al., 1995). Wortmannin and U0126 treatment was performed for 30 min before addition of the Gas6. Thirty microgram of total cell lysates were run on a SDS–PAGE and nitrocellulose subjected to immunoblotting using anti phospho-AKT (Ser473), anti AKT, anti phospho-MAPK, anti p38 and anti p38-MAPK (Cell Signaling, Beverly, MA), anti ERK2 (Santa Cruz, Santa Cruz, CA), anti vinculin, and anti β actin (Sigma). For Gas6 analysis 15 μg of cell extract was used and immunoblot was performed using primary monoclonal anti Human Gas6 IgG antibody (1.5 μg/ml) (R&D Systems, Minneopolis, MN). Membrane was incubated with the appropriated secondary HRP-conjugated IgG antibodies (Amersham Biosciences) and protein was detected with ECL reagent (Amersham Biosciences).

Apoptosis assay

Apoptosis was determined by Fluorescent Terminal Deoxynucleotidyltransferase-Mediated dUTP-Biotin Nick End Labeling (TUNEL). 4×10^4 DU 145 wt and DU 145 Gas6 cells were seeded in 4-well chamber slides. Each assay was performed in two wells and repeated twice. Cells were starved in serum-free RPMI medium with 5 μg/ml of insulin, 5 μg/ml of transferrin, and 4 μM of menadione sodium bisulfite for 24 h. Gas6 was added at 200 ng/ml at 24 and 48 h from seeding. Cells grown in 10% FBS or maintained in serum-free medium were used as controls. Cell permeabilization and the TUNEL reaction were carried out by using the In Situ Cell Death Detection kit (Roche Applied Science) following manufacturer's instructions. Slides washed with PBS were mounted and observed under a fluorescence light microscope (Olympus). For statistic analysis apoptosis was counted in five different microscopic fields (at 400× magnification) for each sample.

Statistical analysis

Mean values ± SD of each group of cases or controls were compared performing Student's *t*-test for independent samples.

RESULTS

Activation of Gas6/Axl signaling induces proliferation on DU 145 and PC-3 cell lines

To analyze if expression of Axl mRNA on DU 145 and PC-3 cell lines has any physiological relevance, we firstly investigated if Axl protein is expressed on these cells. As we can see in Figure 1, immunoblotting analysis shows that Axl protein is differently expressed on these cells: DU 145 show higher expression than PC-3 while Cos-7 resulted negative.

We then examined the relevance of Gas6–Axl interaction on cell growth. We seeded DU 145, PC-3 cells, and

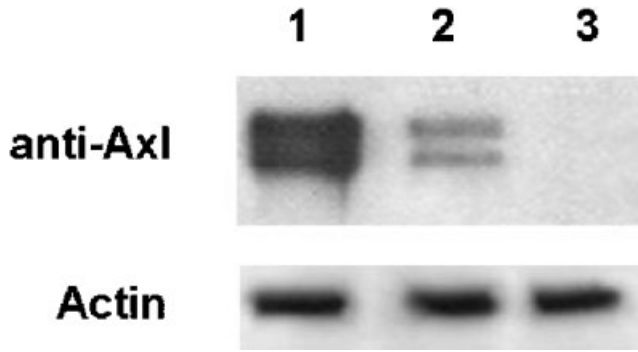

Fig. 1. Immunoblotting analysis of Axl expression. Lane 1: DU 145 wt; lane 2, PC-3 cells; and lane 3, Cos-7 cells.

Cos-7 cells (as a control) in serum-free condition in the presence or absence of different concentrations of Gas6 and evaluated ^{3}HTdR uptake. Figure 2 shows that Gas6 induces proliferation of both DU 145 and PC-3 cell lines, with higher efficiency for the former, while no ^{3}HTdR uptake increase was observed in Cos-7 cells.

Since proliferation assay shows a dose-dependent profile on both DU 145 and PC-3 cell lines, we choose to perform our further studies on Gas6/Axl signaling using 200 ng/ml of Gas6. This concentration is in the same range as the concentration used previously by other investigators which showed mitogenic effect on different targets (Bellosta et al., 1997; Goruppi et al., 2001). To confirm that the mitogenic effect of Gas6 on DU 145 and PC-3 cells is due to Gas6/Axl interaction, we performed ^{3}HTdR uptake experiments incubating cells with Gas6 and a soluble form of Axl receptor, called Axl-X. This protein encodes for the extracellular domain of the receptor which has been shown previously to inhibit Gas6 activity on NIH3T3 cells (Costa et al., 1996). Our results show that soluble Axl-X completely inhibits Gas6 mitogenic effect both on DU 145 ($P < 0.05$) and on PC-3 cell lines ($P = 0.05$) and shows no effects on Cos-7 cells. (Fig. 3).

To confirm that Gas6 mitogenic effect occurs through Axl activation we performed immunoblotting detection of Axl phosphorylation upon Gas6 stimulation. We decided to perform these experiments only on DU 145 cells because the proliferative activity and Axl receptor

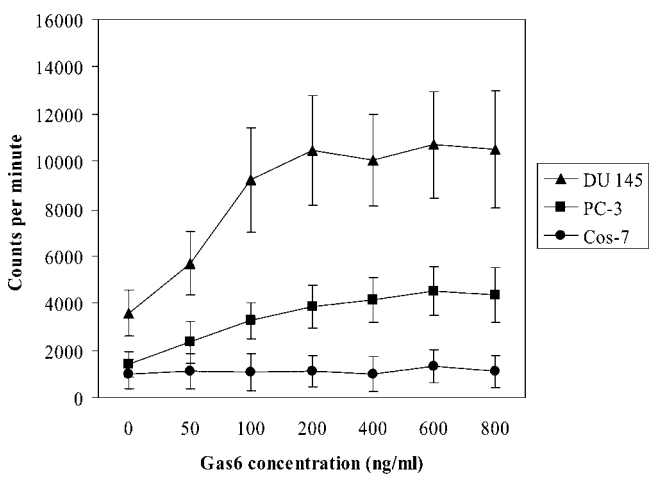

Fig. 2. Gas6 induced proliferation of DU 145 and PC-3 cells. The figure depicts a representative experiment of induction of proliferation by Gas6. Starved cells were stimulated with different concentration of Gas6. Proliferative activity is expressed in counts per minute of ^{3}HTdR incorporation.

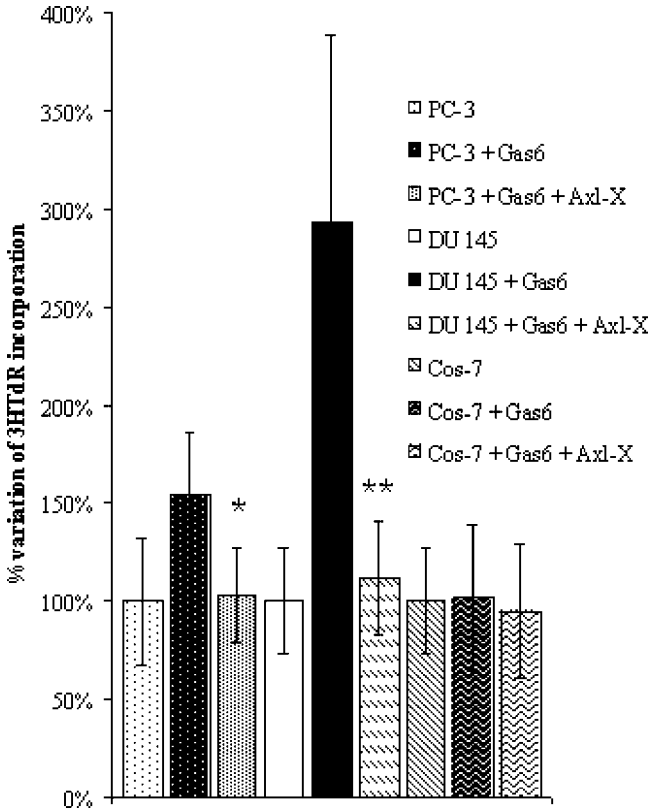

Fig. 3. Axl-X reduces Gas6 induced proliferation of DU 145 and PC-3 cells. Proliferative activity is expressed as percent variation of ^{3}HTdR uptake of unstimulated cells. Axl-X reduces the proliferative activity determined by Gas6 on DU 145 cells ($^{**}P < 0.05$) and on PC-3 cells ($^{*}P = 0.056$).

phosphorylation were stronger in this line compared to that in PC-3 cells. We then analyzed Axl receptor activation in response to Gas6. DU 145 cells were serum starved and then stimulated with 200 ng/ml of Gas6. The level of Axl activation was analyzed by anti-phosphotyrosine immunoblotting. As we can see in Figure 4 stimulation of serum-starved DU 145 cells with 200 ng/ml of Gas6 induces Axl receptor phosphorylation with its maximum activity after 15 min from stimulus declining soon after.

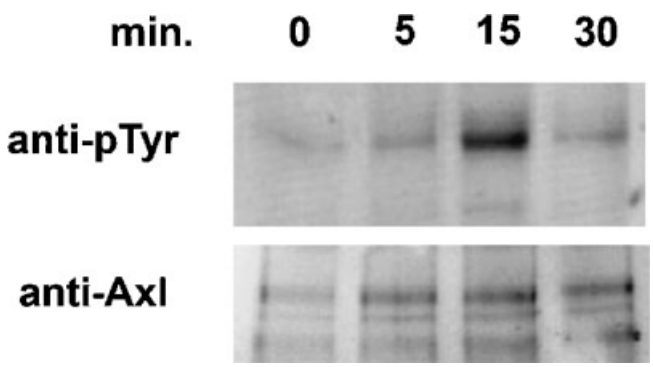

Fig. 4. The Axl receptor is phosphorylated upon Gas6 stimulation in DU 145 cells. Cells were starved in low serum and 200 ng/ml of exogenous Gas6 was added for the indicated time. The Axl receptor was immunoprecipitated from the cell lysate and its level of phosphorylation analyzed by Western blotting using anti-phosphotyrosine antibody. The Axl receptor shows a time dependent phosphorylation, peaking at 15 min after addition of Gas6.

PI3K and MAPK signaling in DU 145 cells

Axl activation is known to produce a signal transduction cascade through different pathways as the PI3K, Src family, and ERK (Braunger et al., 1997). We then wanted to analyze if PI3K and ERK activation are involved in Gas6-induced proliferation of DU 145 cells. Cells were serum starved and stimulated with Gas6 for different times. Then activation of AKT and MAPK was analyzed by Western blot using anti-phospho-specific antibodies. As shown in Figure 5, upon stimulation with Gas6, DU 145 cells show AKT and MAPK phosphorylation peaking at 15 min after stimulus. We then evaluated the contribution to Gas6 induced proliferation of PI3K and ERK signaling through the addition of specific inhibitors of these pathways. The specific inhibitors were added 30 min before adding Gas6, then ^{3}HTdR uptake was performed as described previously. As we can see in Figure 6, the presence of wortmannin (a specific inhibitor of PI3K) reduces Gas6 mitogenic effect by 26%, 44%, and 54% at the concentration of 10 nM, 100 nM, and 1 μM, respectively ($P < 0.05$), while U0126 (a specific inhibitor of MEK1 and MEK2) added at the concentration of 100nM did not influence Gas6 mitogenic effect ($P > 0.05$); but at concentration of 1 μM and 10 μM significantly reduced Gas6 proliferative activity by 47% and 89%, respectively ($P < 0.05$). Since we observed a complete abrogation of the basal proliferative activity of starved DU 145 wt cells by these inhibitors, we wanted to assess if wortmannin and U0126 were toxic to cells.

Wortmannin and U0126 toxicities were analyzed after 24 h of cell starvation by adding 1 μM of wortmannin or 100 μM of U0126 to serum-free RPMI medium. Cells were induced to proliferate by the addition of 1%, 5%, and 10% FBS to the medium and ^{3}HTdR was added for the following 12 h. No statistical difference ($P > 0.05$) was seen between cells treated with wortmannin and

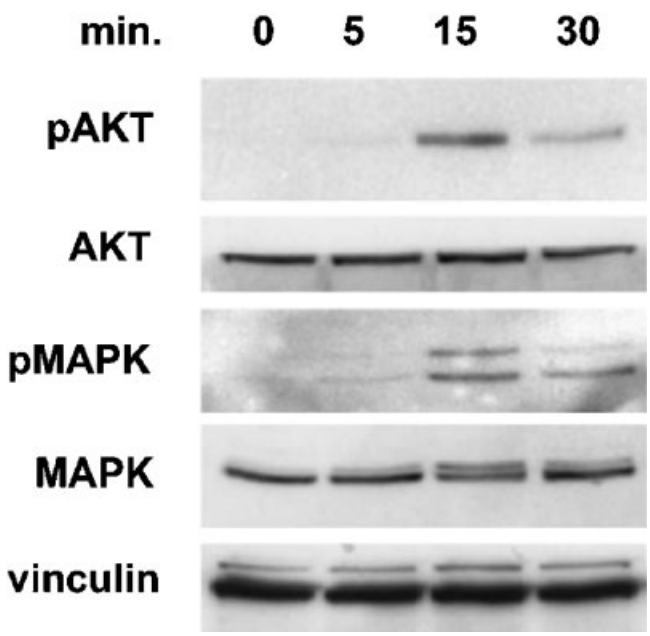

Fig. 5. Gas6/Axl signaling induces AKT and MAPK phosphorylation in DU 145 cells which peaks at 15 min after stimulation. Cells were starved in serum-free condition and 200 ng/ml of Gas6 was added for the indicated time. Total cell lysates were run on a SDS–PAGE and immunoblotted using specific antibodies for phospho AKT or phospho MAPK. Total level of these proteins was analyzed by using anti AKT or anti ERK antisera and anti vinculin as a control for equal loading.

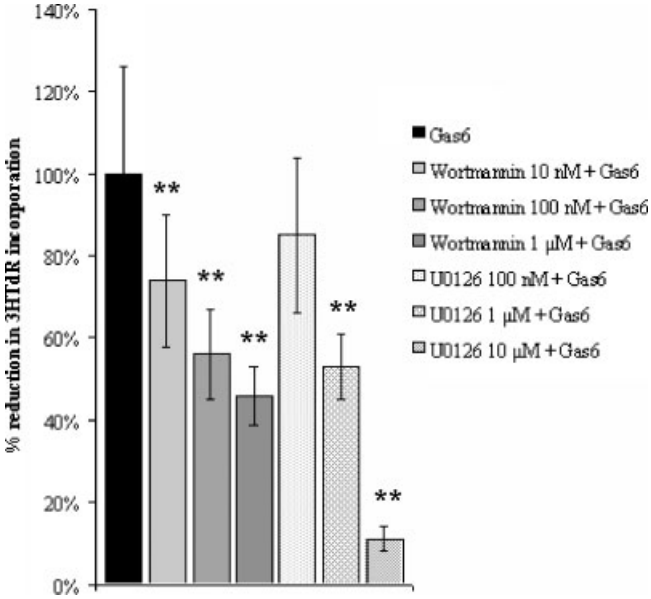

Fig. 6. Wortmannin and U0126 reduce Gas6 proliferative activity on DU 145 cells. Proliferative activity is expressed as percent variation of ^{3}HTdR uptake of stimulated cells, (**$P < 0.05$).

U0126 and those untreated with these inhibitors (data not shown) indicating that wortmannin and U0126 are not toxic to DU 145 wt cells at the concentration tested. We then analyzed the inhibitory effect of wortmannin and U0126 on the phosphorylation of AKT/PKB and on MAPK, respectively. We performed immunoblotting on total lysates of starved DU 145 wt cells stimulated with Gas6. Both AKT and MEK are phosphorylated with a peak at 15 min after Gas6 stimulation (Figs. 5 and 7). Addition of 1 μM wortmannin to serum-free cultured cells before Gas6 stimulation inhibits AKT phosphorylation to basal level, however, we also observed a partial reduction of MEK phosphorylation. Addition of 10 μM U0126 does not affect AKT phosphorylation but completely abolishes MEK phosphorylation (Fig. 7).

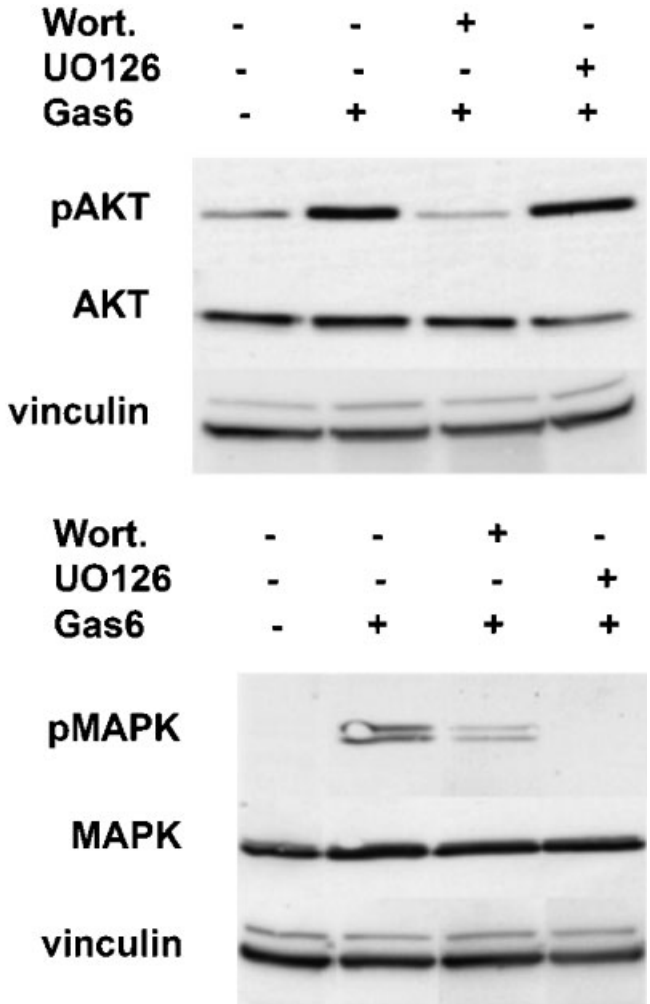

Fig. 7. Inhibition of AKT and MAPK phosphorylation by wortmannin and U0126 in DU 145 cells. Cells were starved in serum-free condition and inhibitors wortmannin (1 μM) and UO126 (10 μM) were added for 30 min before stimulation with Gas6 (200 ng/ml) for 15 min. Total cell lysates were runned on a SDS–PAGE and immunoblotting was performed using specific antibodies for phospho AKT or phospho MAPK. Total level of these proteins was analyzed by using anti AKT or anti ERK antisera and anti vinculin as a control for equal loading.

Effect of the overexpression of Gas6 in DU 145 cells

We transfected human Gas6 in DU 145 cells in order to check if the overexpression of this protein would generate an autocrine loop in these cells determining a proliferative effect comparable to that obtained with exogenous Gas6. To test the success of transfections we performed immunodetection of Gas6 with both immunofluorescence and Western blotting. As shown in Figure 8, immunofluorescence of DU 145 Gas6 cells shows an intense cytoplasmic and membrane fluorescence indicating that these cells are expressing high level of Gas6 protein (Fig. 8 part A) while Mock and wt DU 145 cells show a very low level of expression (Fig. 8 part B and C, respectively), these data were confirmed by Western blotting (Fig. 8 part D) that shows a low production of Gas6 by DU 145 wt and DU 145 Mock in front of higher expression of Gas6 transfected cells, while Cos-7 cells do not show any expression of this protein.

The effect on proliferation of DU 145 Gas6, Mock and wt cells was subsequently analyzed using ^{3}HTdR incorporation assay.

DU 145 Mock cells did not show any statistically significant difference in ^{3}HTdR incorporation with respect to DU 145 wt grown in same conditions ($P > 0.05$).

DU 145 Gas6 cells showed in serum-free condition 166% increase of ^{3}HTdR incorporation rate with respect to wt cells ($P < 0.05$). Moreover, addition of Gas6 (200 ng/ml) to DU 145 Gas6 cells did not increase significantly their mitogenic activity ($P > 0.05$), however, 500 ng/ml Axl-X significantly reduced their proliferation of 65% ($P < 0.05$) (Fig. 9).

Since we observed a small, but consistent, level of proliferation of DU 145 wt cells in serum-free condition and we demonstrated, by immunoblotting analysis, that DU 145 wt cells produce a low level of Gas6, we supposed that the low proliferation effect was mediated by Gas6. To prove our hypothesis we tested the effect of Axl-X in these conditions. As we can see in Figure 10, when the Axl-X was added to the culture medium we observed an inhibition of cell proliferation up to 34% ($P < 0.05$) at the highest dose. Interestingly we also observed an inhibitory effect by wortmannin and U0126 on the basal level of ^{3}HTdR uptake of wt starved unstimulated cells. Wortmannin at a concentration of 10 nM does not influence basal cell growth ($P > 0.05$) but at increased concentrations induces an inhibition of proliferative

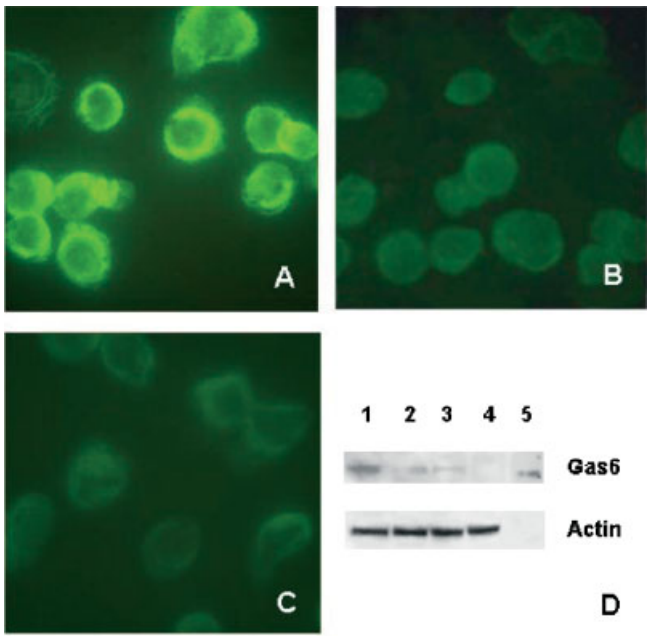

Fig. 8. Gas6 expression in DU 145 transfected cells. **Part A**: Immunofluorescence analysis of DU 145 Gas6, magnification 200×. **Part B** and **C**: Immunofluorescence analysis of DU 145 Mock and wt, respectively. **Part D**: Western blot demonstrating the presence of Gas6 in lysates of different cells; lane 1, DU 145 Gas6; lane 2, DU 145 Mock; lane 3, DU 145 wt; lane 4, COS-7 cells; lane 5, 40 ng of Gas6. Anti β actin antibody was used as control for equal loading.

activity: 100nM and 1μM wortmannin, respectively, reduction of 53% ($P < 0.05$) and 67% ($P < 0.05$) basal ^{3}HTdR incorporation rate. U0126 at 100 nM does not influence DU 145 wt proliferation ($P > 0.05$), however, at 1 μM and 10 μM we observed a reduction of ^{3}HTdR incorporation of 58% and 89%, respectively ($P < 0.05$) (Fig. 11). These results indicate that expression of endogenous Gas6 and its signaling may be responsible for the basal level of proliferation of DU 145 cells in serum-starved condition.

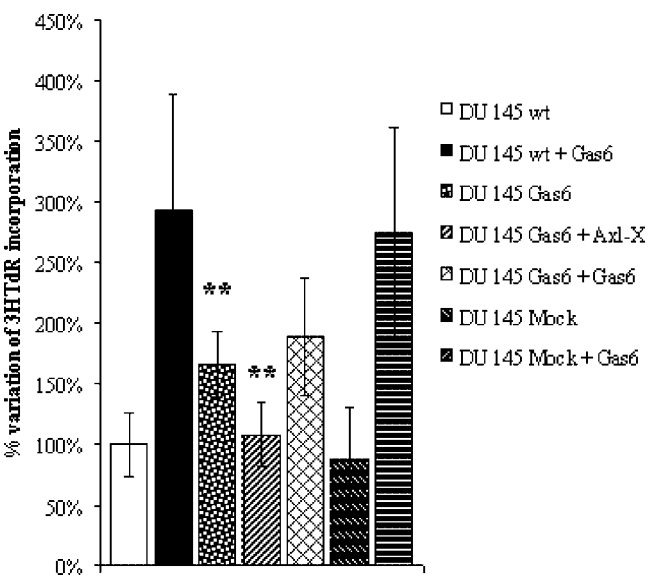

Fig. 9. Axl-x reduces the proliferative activity of DU 145 Gas6 cells. Proliferative activity is expressed as percent variation of ^{3}HTdR uptake of DU 145 wt unstimulated cells. (**$P < 0.05$, see "Results" for details).

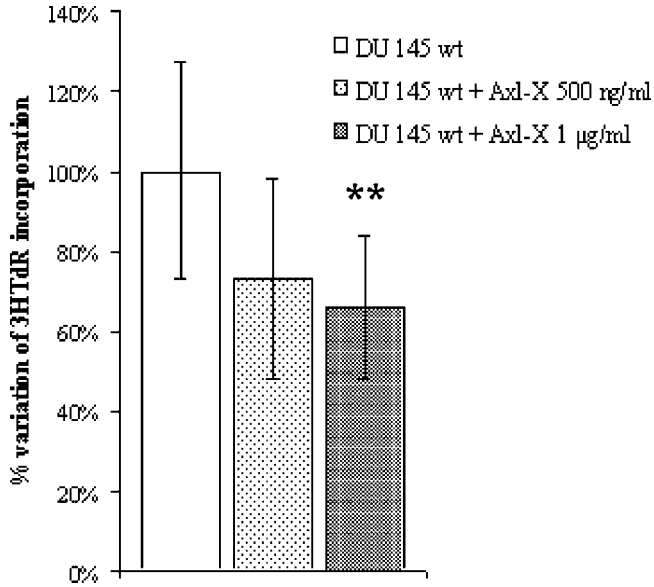

Fig. 10. Axl-X reduces spontaneous proliferative activity of DU 145 wt cells. Proliferative activity is expressed as percent variation of ^{3}HTdR uptake of DU 145 wt unstimulated cells (**$P < 0.05$).

Gas6 does not protect from serum starvation induced apoptosis in DU 145 cells

Since Gas6/Axl activation has been shown to have a predominant role on cell survival, we wanted to analyze if Gas6 was able to protect the DU 145 cells from serum starvation induced apoptosis. We performed TUNEL assay to count apoptotic cells upon addition of Gas6 on starved DU 145 cells. Our results show that only 10% of DU 145 wt cells undergo apoptosis after 4 days of starvation, while in 10% FBS only 0.5%–1% of the cells, show the typical apoptotic pattern. Addition of exogenous Gas6 did not rescue the apoptotic cells indicating that this survival signal is not activated in these

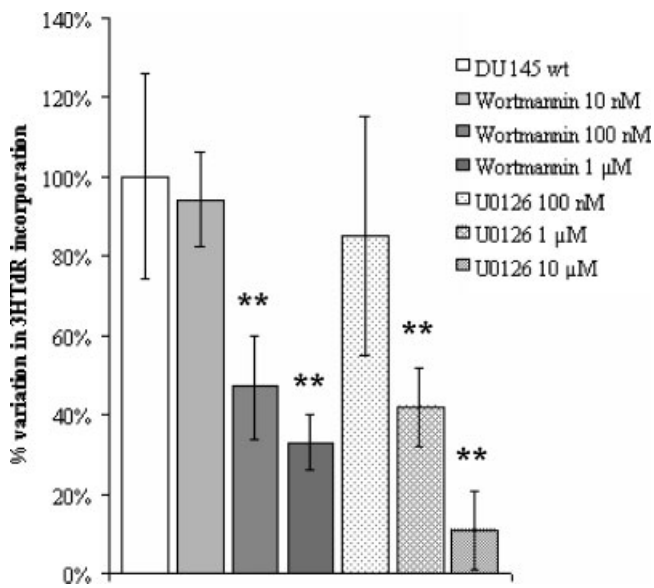

Fig. 11. Wortmannin and U0126 reduce spontaneous proliferative activity on DU 145 wt cells. Proliferative activity is expressed as percent variation of ^{3}HTdR uptake of Gas6 stimulated cells (**$P < 0.05$).

cells. Equivalent results were obtained by comparing DU 145 wt starved and DU 145 Gas6 cells (data not shown).

DISCUSSION

The presence of Axl RTKs family in prostatic cancer has been firstly documented by Robinson et al. (1996) in a prostate carcinoma xenograft CWR22; subsequently, Jacob et al. (1999) demonstrated that the mRNA of Axl was expressed in prostate carcinomas and in cell lines deriving from this type of tumor such as DU 145 and PC-3, however, Axl mRNA is also expressed in normal prostatic tissue. In our study we demonstrate that Axl protein is signaling in both DU 145 and PC-3 cells and that stimulation of the receptor with the recombinant ligand Gas6 determinates proliferation in both cell lines. The entity of the proliferative stimulus, however, is different and proportional to the expression of Axl, being stronger in DU 145 than in PC-3 cells. This observation is of particular interest because it could indicate that the degree of malignancy strictly correlate with Axl expression since DU 145 cells have been described to be more aggressive than PC-3 cells both in vivo and in vitro studies (Webber et al., 2001; van Bokhoven et al., 2003). Previous investigators showed that two signal transduction pathways are mainly involved downstream of Axl signaling: AKT/PI3 kinase pathway and MEK/ERK pathway (Bellosta et al., 1997; Goruppi et al., 1997, 1999, 2001; Allen et al., 1999; Shankar et al., 2003).

AKT protein kinase activation seems to play an important role in prostate carcinogenesis; its constitutive activation highly increases growth in some prostate cancer cell lines like LNCaP (Carson et al., 1999; Davies et al., 1999; Graff et al., 2000), but our and other results (Chen et al., 2001) demonstrated that DU 145 cell line does not have spontaneous constitutive activation of AKT. The *PTEN* (phosphatase and tensine homologue gene) directly inhibits AKT function trough its dephosphorylation (Furnari et al., 1998; Li and Sun, 1998; Ramaswamy et al., 1999; Sun et al., 1999). The *PTEN* gene has been described to show an homozygous deletion or a loss of expression in some prostate cancer derived cell lines, like PC-3 (Li et al., 1997; Vlietstra et al., 1998; Whang et al., 1998; Nakamura et al., 2000) and in prostatic tissues that shows high "Gleason" scores and advanced stage of malignancy (Whang et al., 1998; McMenamin et al., 1999); however, *PTEN* gene function is preserved in DU 145 cell line (Whang et al., 1998; Nakamura et al., 2000; Bastola et al., 2002) and this phosphatase could be important in keeping AKT dephosphorylated (Pfeil et al., 2004). AKT phosphorylation after Gas6 triggering in DU 145 cells could be responsible, at least partially, of Gas6-induced proliferative activity, in fact ^{3}HTdR uptake is 50% reduced by wortmannin that at the same dose almost completely inhibits AKT phosphorylation by Gas6. On the other hand the low level of proliferation induced by Gas6 in PC-3 cells could be interpreted either because of the low level of the Axl protein or, alternatively, to the fact that since AKT is constitutively activated in this cell line, due to a non functional mutation of *PTEN* (Pfeil et al., 2004), the triggering of Axl by Gas6 may not be able to induce further AKT phosphorylation. This interpretation is confirmed by the results obtained with IGF-1 treatment on DU 145, PC-3, and LNCaP cells (the last showing the absence of *PTEN* product) (Nakamura et al., 2000; Pfeil et al., 2004). Upon IGF-1 treatment, DU 145 cells proliferate and show AKT phosphorylation while in the same condition PC-3 and LNCaP cells do not respond to IGF-1 proliferation. These data will suggest that the AKT pathway is already fully turned on in these cells (Pfeil et al., 2004).

Because most studies of AKT have focused on its role in cell survival and many evidences in the literature emphasize an anti-apoptotic effect of Gas6 on several cell lines (Nakano et al., 1995; Avanzi et al., 1997; Bellosta et al., 1997; Goruppi et al., 1997; Allen et al., 1999), we wanted to investigate if the growth effect mediated by Gas6 in DU 145 cells could be due to an inhibition of the apoptotic process. As we were not able to show any survival effect of Gas6 after 4 days of serum starvation of DU 145 cells, we conclude that this survival pathway may be absent or impaired in these cells. By the other hand it has been demonstrated that this cell line has an extremely low kinetic of apoptosis upon serum withdrawal (Tang et al., 1998), possibly due to the lack of Bax, an essential component in the cell death activation machinery or because of overexpression of Bcl-2 family of anti-apoptotic proteins (Tang et al., 1998; Rothermund et al., 2002; Chiu et al., 2003). Taken together, our data demonstrate that the proliferative activity mediated by Gas6 on DU 145 cells is, at least partially, mediated by AKT phosphorylation and does not involve survival process. This is not surprising because it is well known that AKT can also effect proliferation through signals to cell-cycle machinery. It has an important role in preventing cyclin D1 degradation by regulating the activity of GSK3β. AKT directly phosphorylates GSK3β and blocks its kinase activity, thereby allowing cyclin D1 to accumulate and induce G1/S phase transition (Diehl et al., 1998). GSK3β activation has been demonstrated in DU 145 cells upon stimulation with IGF-1 and EGF (Pfeil et al., 2004) and in C57MG mammary cells triggered by Gas6 (Goruppi et al., 2001).

However, other pathways beyond AKT could be involved, by Gas6/Axl interaction in our model. We demonstrated that MEK inhibitor U0126 affects p44/42 MAPK phosphorylation upon Gas6 stimulation of DU 145 cells while no phosphorylation of the p38-MAPK was found (data not shown). Moreover U0126 at concentration of 10 μM significantly reduced Gas6 proliferative activity by 89%. These results suggest that PI3K contribute only partially to the Gas6/Axl mediated mitogenic effect, and that Gas6 mediated proliferation also requires RAS-MAPK pathway.

Another point of interest is the biological role of Axl overexpression in these metastatic prostate cancer cell lines. We demonstrated that Gas6/Axl interaction is able to increase cell proliferation in serum-free condition, thus Axl overexpression could be simply considered a molecular event that contribute to cell proliferation, in particular unfavorable conditions such as the presence in a different tissue. This hypothesis could be sustained by the fact that these cell lines have been established from metastatic prostate cancers. But other interpretations are possible. Axl protein has been described to be involved in intercellular binding through homophilic interaction between cells expressing Axl receptor (Bellosta et al., 1995; Avanzi et al., 1998); considering that metastatic process occurs through embolization of aggregates of cancer cells and these cells adhere to endothelium through adhesion molecules (Orr et al., 2000; Bogenrieder and Herlyn, 2003), we can make an hypothesis about a role of Axl in this process as follows: Axl could promote cells aggregation in bloodstream avoiding cell dispersion and/or Axl could have a role in cells adhesion to endothelium which highly express Axl

(Avanzi et al., 1998). On the other hand Gas6 could play an antagonist role interposing itself between Axl/Axl interaction inducing disaggregation of cancer cells (Avanzi et al., 1998). Further experiments in this direction are needed to confirm these hypotheses.

In conclusion, we demonstrated a mitogenic activity determined by Gas6/Axl interaction in undifferentiated metastatic human prostatic cancer cell lines. This effect is proportional to Axl expression, not due to inhibition of apoptosis and induces AKT and MAPK phosphorylation.

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