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Requirement of Phosphatidylinositol 3-Kinase-Dependent Pathway and Src for Gas6-Axl Mitogenic and Survival Activities in NIH 3T3 Fibroblasts

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Gas6 is a secreted protein previously identified as the ligand of the Axl receptor tyrosine kinase. We have shown that Gas6 is able to induce cell cycle reentry of serum-starved NIH 3T3 cells and to efficiently prevent apoptosis after complete growth factor removal, a survival effect uncoupled from Gas6-induced mitogenesis. Here we report that the mitogenic effect of Gas6 requires phosphatidylinositol 3-kinase (PI3K) activity since it is abrogated both by the specific inhibitor wortmannin and by overexpression of the dominant negative PI3K p85 subunit. Consistently, Gas6 activates the PI3K downstream targets S6K and Akt, whose activation is abrogated by addition of wortmannin. Moreover, rapamycin treatment blocks Gas6-induced entry into the S phase of serum-starved NIH 3T3 cells. We also demonstrate the requirement of Src tyrosine kinase for Gas6 signalling since stable or transient expression of a catalytically inactive form of Src significantly inhibited Gas6-stimulated entry into the S phase. Accordingly, Gas6 addition to serum-starved NIH 3T3 cells causes activation of the intrinsic Src kinase activity. When specifically analyzed in a survival assay, these elements were found to be required for the survival effect of Gas6. Taken together, the evidence presented here identifies elements involved in the Gas6 transduction pathway that are responsible for its antiapoptotic effect and suggests that Src is involved in the events regulating cell survival.

The protein encoded by *gas6* (Gas6) is a growth factor member of the vitamin K-dependent family with a significant degree of amino acid identity to protein S (42). Both human and murine *gas6* cDNA have been characterized, and a potentiating growth factor has been shown to encode the rat Gas6 homolog (45). Gas6 has been shown to bind and activate the receptor tyrosine kinase Axl (65), its C-terminal region being responsible for such interaction (42a, 43).

Axl is the founder of a family of tyrosine kinase receptors (47). Members of this receptor family have been isolated in humans (c-Mer/Nyk [28, 40] and Sky/Rse [44, 48]), mice (Brt [23]), and chickens (Eyk [33]). Murine and rat homologs of Axl were also identified and named Ark and Tyro7, respectively (38, 53). Analysis of Ark and Ufo mRNA expression in murine tissues revealed a near-ubiquitous expression, with the highest levels found in the breast, ovary, spleen, bone marrow, heart, and brain (32).

Experimental evidence obtained by Godowsky et al. demonstrated that Gas6 is able to activate the Axl-related Rse/Sky tyrosine kinase receptor (24). This observation clarified the issues raised in a previous report indicating protein S as the ligand for the Rse/Sky receptor (60). As suggested previously (24), such differences should be ascribed to the different affinities in interspecies ligand receptor recognition. Consistently Gas6, but not protein S, was recently reported to bind and activate Sky tyrosine kinase activity (49).

When quiescent cells are stimulated with a variety of growth factors, the phosphatidylinositol 3-OH kinase (PI3K) is activated and becomes associated with several proteins including growth factor receptors and nonreceptor tyrosine kinases of the Src family, Crk and Abl (7, 64). PI3K is a heterodimer of the p85 subunit and the p110 subunit with catalytic activity; p85 is responsible for specific interactions, e.g., with activated receptors (15). By using either specific inhibitors or constitutively active and dominant negative mutants, a role for PI3K has been established in mitogenic signalling, apoptosis, intracellular vesicle trafficking, and cytoskeletal regulation (6, 8, 54, 57). Consistently, the PI3K inhibitor wortmannin causes apoptosis in PC12 (66) and interference with ruffle formation (8).

A well-characterized downstream molecular target for PI3K is the mitogen-regulated kinase specific for ribosomal protein S6 (S6K) (11). S6K is rapidly activated by growth factors, stress inducers such as anysomicin, and oncogene products (35, 51). Multiple lines of evidence indicate that S6K plays an important role during the G_0-to-S-phase transition. Inhibition of S6K activation by antibody microinjection or via the specific inhibitor rapamycin significantly interferes with the G_0-to-S entry by inducing G_1 arrest or delaying S-phase entry (12, 36).

More recently, the existence of a specific pathway involving PI3K and S6K distinct from the traditional growth factor pathways (13, 16) has been sustained with the characterization of the serine/threonine kinase Akt (3, 6). Akt has low catalytic activity in serum-starved cells, which is rapidly stimulated by the addition of various growth factors. Its activation is blocked by the PI3K inhibitor wortmannin (21), but it is insensitive to rapamycin. Taken together, these findings locate Akt between PI3K and S6K. The complexity of the growth factor response in quiescent cells was recently increased by findings indicating the existence of an Src-specific pathway, regulating *myc* transcription, that is required for DNA synthesis in response to platelet-

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derived growth factor (PDGF), epidermal growth factor (EGF) and colony-stimulating factor type 1 (1).

Three highly related members of the Src family of tyrosine kinases, Src, Fyn, and Yes, are expressed by NIH 3T3 cells (37, 55). Transient activation of Src intrinsic kinase activity and increase of Src protein phosphorylation in quiescent fibroblasts are stimulated by mitogens, cytokines, cell-matrix interactions, and oxidative stress as induced by UV irradiation (18). The requirement of Src during the G_0-to-S-phase transition induced by PDGF and EGF was demonstrated by using either a dominant negative, kinase-inactive Src or antibodies that inhibit Src, Fyn, and Yes (55, 62).

We previously reported that Gas6 is able to induce cell cycle reentry of serum-starved NIH 3T3 and to efficiently prevent apoptosis as induced by the complete withdrawal of serum. In this study, we have analyzed the signal transduction elements required for Gas6/Axl mitogenic and survival effects. We found that PI3K, S6K, and Src are required for both Gas6-induced mitogenesis and its antiapoptotic effect.

MATERIALS AND METHODS

Plasmids. pSG5 plasmids containing human c-Src K+ and dominant negative c-Src K− cDNA were kindly provided by S. Courtneidge. cDNA encoding the wild-type p85 subunit of PI3K and the dominant negative Δ110 deletion mutant were kindly provided by S. Volinia, University of Ferrara. All the relevant cDNA inserts were subcloned in the pGDSV7 eucaryotic expression vector (14) when specified.

Cell culture. NIH 3T3 and COS-7 cell lines were grown routinely in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). For growth arrest by serum starvation, NIH 3T3 cells were seeded at 10^4 cells per cm^2, and after 24 h of plating in 10% FCS, the medium was replaced with DMEM containing 0.5% FCS and the incubation was continued for 48 h. After this time, incubation for 1 h with 50 μM bromodeoxyuridine (BrdU) (Fluka) resulted in less than 5% of BrdU-positive nuclei. Wortmannin, rapamycin, and sodium orthovanadate were purchased from Sigma.

Cell lines stably expressing Src were obtained by cotransfecting NIH 3T3 cells with pSG5 Src K− and pDNAI plasmids. Selection was performed by culture in 1 mg of G418 (Gibco) per ml. G418-resistant colonies were clonally expanded, and Src expression was analyzed by Western blotting of total-cell lysates with 327 antibody (41).

DNA synthesis assay. The ability to induce DNA synthesis was tested by adding the indicated growth factors directly to cells in the starvation medium together with 50 μM BrdU. After 22 h, the cells were fixed with 3% paraformaldehyde (Fluka) in phosphate-buffered saline (PBS) and processed for immunofluorescence as described previously (25). For immunofluorescence, mouse monoclonal antibody immunoglobulin G2a (IgG2a) anti-BrdU (Amersham, Little Chalfont, United Kingdom) was used followed by a second antibody, rhodamine isothiocyanate (RITC)-conjugated anti-mouse IgG2a (Southern Biotechnology). Total nuclei were visualized by staining with Hoechst 33342 dye (2 μg/ml in PBS) for 5 min, and the coverslips were mounted with Moviol mounting solution before analysis. The percent S-phase induction was calculated from the ratio between nuclei positive for RITC (BrdU) and total nuclei (Hoechst). Basic fibroblast growth factor (bFGF) was kindly supplied by C. Grassi-Farmitalia. Recombinant Gas6 and recombinant PDGF were supplied by Amgen Inc. For analysis of interference with the S-phase induction, quiescent NIH 3T3 cells were pretreated with various concentrations of drugs. Wortmannin (Sigma) was added at 10, 1, 0.1, 0.01, and 0.001 μM for 30 min and rapamycin (Sigma) was added at a 20-ng/ml final concentration for 20 min before growth factor and BrdU addition (50 μM).

All experimental results were analyzed with a Zeiss microscope with the following filters: rhodamine BP 546, FT 580, LP 590; fluorescein BP 450-490, FT 510, LP520, Hoechst G 365, FT 395, LP 420.

Western blot analysis. For analysis of p85 association with the Axl receptor, serum-starved NIH 3T3 cells in a 10-cm petri dish were stimulated or not for 10 min with 400 ng of Gas6 per ml and lysed in 1 ml of ice-cold lysis buffer (150 mM NaCl, 50 mM Tris [pH 7.5], 1% Triton X-100, 5 mM EDTA, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, CLAP at 10 μg/ml [aprotinin, leupeptin, antipain, and pepstatin, all from Boehringer Mannheim]). Equal amounts of cell lysates were incubated with 3 μg of affinity-purified anti-Axl antibody for 3 h at 4°C and then with 15 μl of Ultralink protein A (Pierce) for a further 1 h. Immunocomplexes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) and blotted to a nitrocellulose membrane. When GSTp85 was used, lysates of stimulated and unstimulated cells were incubated for 1 h at 4°C with 2 μg of baculovirus-expressed GSTp85 prebound to glutathione-Sepharose (Pharmacia) (a gift from S. Volinia). The proteins bound to the beads were washed three times in lysis buffer, resolved by SDS-PAGE, and blotted to a nitrocellulose membrane. Western blotting was carried out with a monoclonal anti-p85 isoform beta for anti Axl immunoprecipitations and rabbit polyclonal antibody anti-Axl for GSTp85 (15, 17). Complexes were visualized with peroxidase-conjugated second antibodies (Southern), goat anti-mouse and goat anti-rabbit respectively, with enhanced chemiluminescence (ECL) solutions (Amersham).

Analysis of mitogen-activated protein kinase (MAPK), S6K, and Akt activation was performed 10 min after the addition of growth factors to growth-arrested NIH 3T3 cells. Gas6 (400-ng/ml final concentration) was added to 6-cm-diameter petri dishes. As a control, in separate experiments, cells were stimulated with 100 ng of bFGF per ml or left untreated. After this time, the cells were washed twice in PBS–1 mM sodium orthovanadate at 4°C and lysed in SDS sample buffer containing 1 mM sodium orthovanadate. Loading of equal amounts of total proteins was assessed by Coomassie staining of separate gels. After SDS-PAGE (10% polyacrylamide) and transfer to a polyvinylidene difluoridene membrane (Millipore), Western blot analysis was carried out by using anti-MAPK, anti-S6K, or anti-Akt1 rabbit polyclonal antibody (Santa Cruz Laboratories) and visualized by using a goat anti-rabbit peroxidase-conjugated second antibody (Southern) and ECL (Amersham).

When wortmannin (1 μM) or rapamycin (20 ng/ml) was used, it was added to the starvation medium (for 30 and 20 min, respectively) prior to activation.

To analyze c-*src* activation, COS-7 cells were transfected with pSG5 Src K+ cDNA by the DEAE-dextran method, and a separate petri dish was mock transfected as a control. The following day, cells were serum starved (0.5% FCS) for 24 h and then stimulated for 10 min with 400 ng of Gas6 per ml or left untreated. The cells were lysed in SDS-PAGE loading buffer containing 1 mM sodium orthovanadate and equal amounts of proteins (as assessed by Coomassie staining of separate gels) and separated by SDS-PAGE (10% polyacrylamide). After blotting to nitrocellulose membrane, Western blot analysis was carried out by using PY 20 anti-phosphotyrosine antibodies (Transduction Laboratories) and ECL solutions (Amersham). As control, a separate membrane was decorated with anti-c-*src* 327 monoclonal antibodies.

Analysis of Gas2 cleavage after cell death induction in serum-starved NIH 3T3 and GSR4 cells was performed with an anti-Gas2-specific antibody (4) essentially as described previously (26). A 10-cm petri dish of serum-starved NIH 3T3 cells was washed with PBS and incubated for 24 h in serum-free DMEM containing or not containing either 400 ng of Gas6 per ml or 100 ng of bFGF per ml as a control. Then both adherent and nonadherent cells were combined, and SDS lysates were separated by SDS-PAGE and analyzed by Western blotting. Equal loading of proteins was assessed with separate gels stained with Coomassie blue.

Src kinase assay. Serum-starved NIH 3T3 cells in a 15-cm petri dish were stimulated for 0, 10, 20, or 30 min with 400 ng of Gas6 per ml. The cells were lysed in 1.8 ml of ice-cold lysis buffer (150 mM NaCl, 50 mM Tris [pH 7.5], 1% Triton X-100, 5 mM EDTA, 2 mM sodium orthovanadate, 1 mM PMSF, 10 μg of CLAP per ml). Cell lysates were transferred to Eppendorf tubes, vortexed, incubated for 10 min, and then centrifuged for 15 min at 10,000 × *g* in an Eppendorf centrifuge to remove insoluble material. The supernatants were precleared by incubation for 30 min with protein A-Sepharose (Pharmacia) (10% [vol/vol] final concentration) and then incubated on ice with 2 μl of anti-c-*src* polyclonal antiserum (67) for 60 min. After this time, the lysates were transferred to Eppendorf tubes containing 10 μl of protein A-Sepharose (previously washed in lysis buffer) and incubated for 45 min at 4°C on a rotating platform. Immunoprecipitates were washed three times in lysis buffer and twice in lysis buffer without Triton X-100 or EDTA. Kinase assays were performed for 15 min at 30°C in 15 μl of 20 mM Tris (pH 7.5)–20 mM $MnCl_2$–1 mM $CaCl_2$ containing 3 μCi of [γ-^{32}P]ATP (370 MBq/mmol; Amersham). The reactions were terminated by adding 3 μl of 5× SDS-PAGE loading buffer, and the products were resolved by SDS-PAGE (10% polyacrylamide). The dried gel was exposed for 2 h at room temperature with intensifying screens (Dupont Cronex).

Microinjection. All experiments were performed with an automated injection system (AIS; Zeiss, Oberkochen, Germany). Plasmid DNA dilutions in double-distilled water were loaded into 1.2-mm-diameter glass capillaries (Clark Electro Medical Instruments, Reading, United Kingdom) pulled with a capillary puller (Mecanex S. A., Geneva, Switzerland). NIH 3T3 cells, growth arrested by serum starvation, were microinjected with pGDSV7-p85 (20 ng/μl), pGDSV7-p85Δ110 cDNA (20 ng/μl), pGDSV7-TrR (20 ng/μl) (14), or pSG5 Src K− (10 ng/μl) cDNA. At 4 h after microinjection, Gas6 or different growth factors as indicated were added to the cells together with 50 μM BrdU. The cells were fixed, and double immunofluorescence was performed with an IgG1 monoclonal antibody, anti-p85 (17); 327 monoclonal antibody anti-c-Src (41); and a commercial monoclonal IgG2a anti-BrdU (Amersham). The second antibodies were RITC-conjugated anti-mouse IgG2a (Southern Biotechnology) and fluorescein isothiocyanate-labeled goat anti IgG1 (Southern Biotechnology). Total nuclei were visualized with Hoechst 33342 dye (2 μg/ml in PBS). Incorporation of BrdU into control cells was evaluated by using fields of uninjected cells. Immunofluorescence results were analyzed with an inverted microscope (Axiovert; Zeiss), and images were collected with a confocal laser scan microscope (Zeiss).

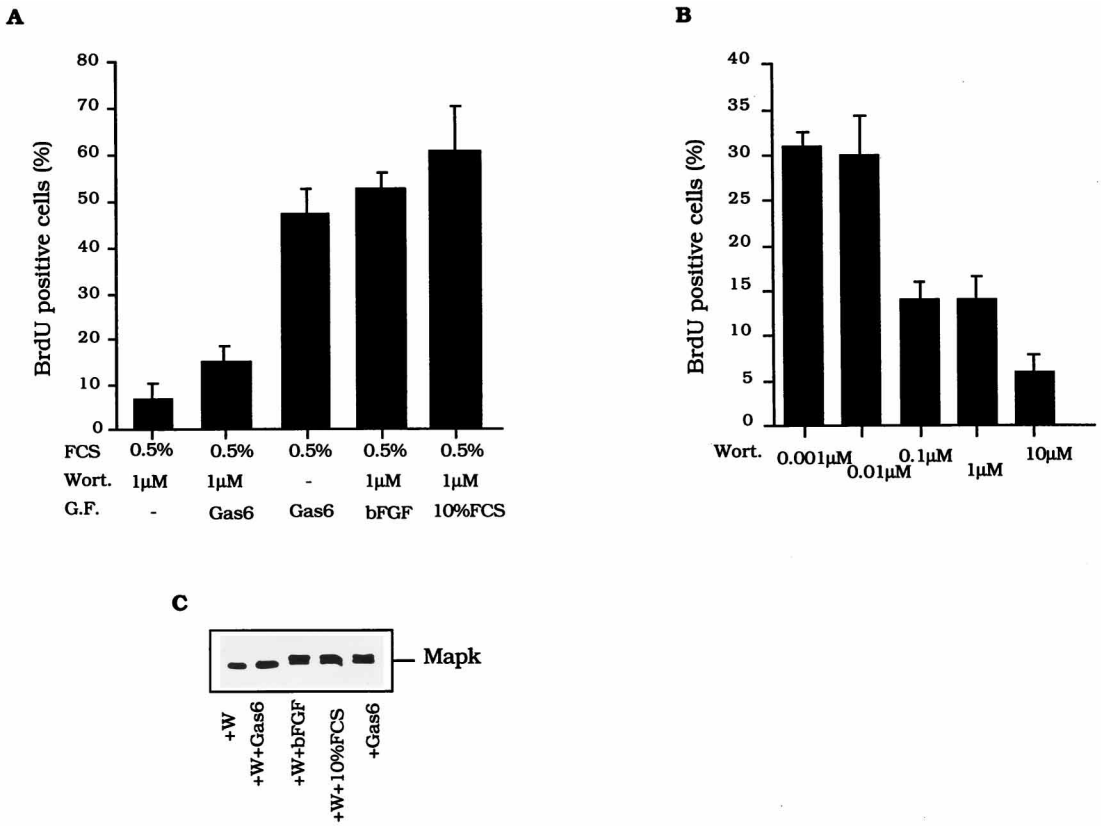

FIG. 1. Wortmannin blocks the mitogenic effect of Gas6. (A) Analysis of S-phase induction in serum-starved NIH 3T3 cells in the presence of wortmannin. Serum-starved NIH 3T3 cells (0.5% FCS for 48 h) were treated with 1 μM wortmannin (Wort.) for 30 min before being stimulated with 400 ng of Gas6 per ml, 100 ng of bFGF per ml, or 10% FCS. A petri dish was left untreated and similarly stimulated with Gas6 as a control. DNA synthesis was monitored by adding 50 μM BrdU together with the indicated growth factor. Cells were fixed after 22 h and processed for immunofluorescence as described previously (25). The percent BrdU incorporation was calculated as the ratio between nuclei positive for RITC (BrdU) and total nuclei (Hoechst). The mean of four independent experiments and the standard deviation of the mean are reported. (B) Wortmannin dose-response analysis for inhibition of Gas6-induced entry into the S phase. Different concentrations of wortmannin (0.001, 0.01, 0.1, 1, and 10 μM) were added to serum-starved cells before stimulation with Gas6. DNA synthesis was analyzed as described in panel A. After Gas6 addition, 46% ± 6% of the cells entered the S phase in this set of experiments. The mean and the standard deviation of the mean of three separate experiments are shown. (C) Wortmannin blocks MAPK activation by Gas6. Serum-starved NIH 3T3 cells were treated with 1 μM wortmannin (+W) before stimulation with Gas6, bFGF, or 10% FCS for 10 min. A separate petri dish was not treated with wortmannin and was similarly stimulated with Gas6 as positive control (+Gas6). Western blot analysis was performed on total cellular lysates with anti-Erk2 polyclonal antibodies as previously reported (26).

RESULTS

Wortmannin blocks Gas6-induced mitogenic activity. Most receptor tyrosine kinases activate the PI3K pathway through recruitment of PI3K, an enzyme with a regulatory p85 subunit and a catalytic p110 subunit (7). Analysis of the amino acid sequence of the Axl cytoplasmic region showed two sites with the consensus sequence required for a PI3K binding (YXXM) (47, 59). To have further insights in the downstream components that are responsible for the Axl signalling, we analyzed the effect of wortmannin, a potent PI3K inhibitor (63), on Gas6-induced mitogenic activity.

Serum-starved NIH 3T3 cells were pretreated for 30 min with 1 μM wortmannin before the addition of Gas6 (400 ng/ml, final concentration), bFGF (100 ng/ml), or serum (10% FCS). As control, Gas6 mitogenic activity in the absence of drug was measured in a parallel experiment. Mitogenic assays were carried out essentially as described previously (26): cells were allowed to incorporate BrdU for 20 h before immunofluorescence staining. Wortmannin addition completely abolished Gas6-induced mitogenic activity (Fig. 1A) with no significant effect on either bFGF or 10% FCS used as controls (see the figure legend). We next analyzed whether lower concentrations of wortmannin could inhibit Gas6 mitogenic activity: the 50% inhibitory concentration for inhibition of Gas6-induced S-phase entry was less than 10 nM in vivo (Fig. 1B), similar to that described for inhibition of enzyme activation by PDGF (13) and HGF/SF (56) both in vitro and in vivo.

We previously reported that Gas6 stimulation of serum-starved NIH 3T3 cells induces activation of the MAPK pathway (26) (and Fig. 1C). We therefore investigated whether pretreatment with wortmannin was able to block the activation of such a downstream molecular target. Serum-starved NIH 3T3 cells were pretreated with 1 μM wortmannin before being stimulated for 10 min with Gas6, and the lysates were analyzed by Western blotting with anti-MAPK antibodies (Fig. 1C). Separate petri dishes were stimulated in parallel experiments with bFGF and FCS as controls. Consistent with the results obtained for the mitogenic assays, wortmannin treatment abrogated Gas6-induced MAPK activation, as evidenced by the absence of electrophoretic lower-mobility shift, while having no such effect on bFGF or FCS. These results suggested the requirement of an active PI3K for Gas6-induced mitogenesis.

Binding of p85 to endogenous Axl receptor. A critical step in PI3K regulation is the association of the p85 subunit with the growth factor receptors (64). We further assessed whether Gas6 stimulation results in the association of p85 with Axl

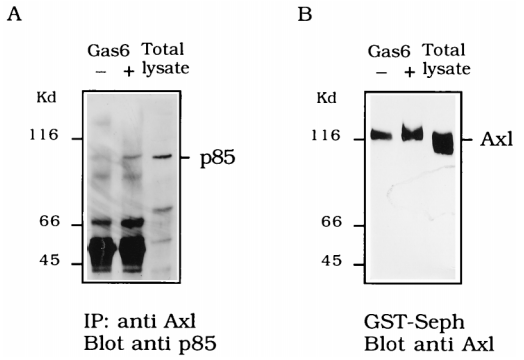

FIG. 2. p85 associates with Axl receptors. (A) Gas6 induces p85 binding to Axl. Serum-starved NIH 3T3 cells were stimulated with 400 ng of Gas6 per ml for 10 min (+) or left untreated (−), and the resulting cellular lysates were immunoprecipitated with anti-Axl antibodies. The immunocomplexes were resolved by SDS-PAGE (10% polyacrylamide), and Western blotting was carried out with anti-p85 monoclonal antibody. An aliquot of unstimulated total cellular lysate was also analyzed to monitor p85 protein expression. (B) Gas6 treatment increases the association of Axl with p85. Cells were stimulated (+) or left untreated (−) as in panel A, and equal amounts of total proteins were incubated with GSTp85 for 1 h. Bound proteins were separated by SDS-PAGE (10% polyacrylamide), and immunoblots were decorated with anti-Axl antibodies. The total-cell lysate was similarly analyzed for Axl protein expression.

receptors. Serum-starved NIH 3T3 cells were stimulated with 400 ng of Gas6 per ml for 10 min or left untreated, and Axl receptors were immunoprecipitated from the resulting cell lysates with anti-Axl antibodies. After electrophoresis and blotting, the membrane was revealed with anti-p85 monoclonal antibodies. A marked increase in p85 association with Axl receptors was observed after Gas6 addition (Fig. 2A), consistent with the findings obtained with an EGFR-Axl chimeric receptor in a 32D cell system (22).

This association was further investigated with a baculovirus-expressed GST-p85 protein. Serum-starved NIH 3T3 cells were treated as above, and the cell lysates were incubated with glutathione-Sepharose GST-p85 for 1 h. After this time, the beads were extensively washed and the bound proteins were analyzed by Western blotting with anti-Axl antibodies. Figure 2B shows that upon Gas6 addition, the amount of endogenous receptors that associate with GST-p85 is increased. However, Axl receptors were also present in nonstimulated complexes; since no Axl association was found when GST alone was used as a control (not shown), these data could suggest a significant basal constitutive activation of p85 target sites in Axl receptors. Accordingly, p85 was reported to associate with Axl receptors in a similar manner in an Axl-expressing 32D cell line (22). These results indicated that addition of Gas6 to serum-starved cells increases p85 association with Axl receptors.

Gas6 induced DNA synthesis requires PI3K. To further examine the interaction between PI3K and Axl receptor, we asked whether expression of a dominant negative p85 protein was able to interfere with Gas6 mitogenic activity. As a dominant negative protein, we used the construct p85Δ110, a deletion mutant defective for association with the p110 subunit (15, 29). The cDNAs coding for wild-type p85 and p85Δ110 (kindly provided by S. Volinia) subcloned in a simian virus 40 promoter expression vector were microinjected into the nucleus of serum-starved NIH 3T3 cells. At the indicated times, the cells were stimulated with Gas6 or FCS as a control and subsequently fixed and analyzed by double immunofluorescence for p85 overexpression and BrdU incorporation. Overexpression of p85Δ110 significantly inhibited Gas6-induced cell cycle reentry (11.5% ± 2.5% compared to 49.7% ± 4.6% in uninjected cells) (Fig. 3B), whereas wild-type p85 microinjection had no significant effect (46.5% ± 3.2% compared to 48.9% ± 6% in uninjected cells). Cells stimulated in parallel experiments with 10% FCS, which is not antagonized by p85Δ110, showed no significant inhibition (Fig. 3). These data confirmed the strict requirement for an active PI3K in the Gas6-stimulated mitogenic signal.

Gas6 mitogenic activity requires S6K. Gas6-induced mitogenic activity was further characterized by investigating the role of S6K, a known downstream target of PI3K (11). We used the macrolide rapamycin, a potent and specific inhibitor of S6K activation (12, 36). NIH 3T3 cells were serum starved and incubated with rapamycin (20 ng/ml, final concentration) for 20 min. After this time, 400 ng of Gas6 per ml, 100 ng of bFGF per ml, or 10% FCS was separately added together with BrdU (50 μM), and the cells were subjected to a mitogenic assay as previously described. Figure 4A shows that inhibition of S6K activation by rapamycin completely abolished Gas6-induced cell cycle reentry. Conversely, no significant effect was detected when cells were stimulated by either bFGF or FCS, suggesting that Gas6 requires activation of S6K to induce entry into the S phase.

S6K has a basal phosphorylation level in quiescent fibroblasts and undergoes multiple and complex phosphorylations on novel sites after mitogenic stimulation (35). Since rapamycin is highly specific for S6K, although it is not the direct target (11), we analyzed the ability of Gas6 to induce phosphorylation of S6K. NIH 3T3 cells were serum starved and pretreated for 20 min with 20 ng of rapamycin per ml before stimulation for 10 min with Gas6, bFGF, or FCS, at similar concentrations to those used for the mitogenic assays. In a parallel experiment, cells were similarly stimulated with no drug addition, and one petri dish was left untreated as a control. The cells were lysed by adding SDS loading buffer to the petri dishes, and Western blot analysis of total proteins was carried out with anti-S6K-specific antibodies. Gas6 addition to serum-starved cells induced the activation of S6K, evidenced as lower-mobility electrophoretic bands, to a comparable extent to that seen in the positive controls bFGF and 10% FCS (Fig. 4B). Consistent with the results obtained in the mitogenic assay, pretreatment with rapamycin abrogated S6K activation by Gas6. Conversely and as previously reported (11), rapamycin inhibited bFGF and FCS activation of S6K without significantly affecting their ability to promote S-phase entry (as also shown previously). These results indicated that Gas6 requires activation of S6K to carry out its mitogenic activity in quiescent NIH 3T3 cells.

Gas6 requires the PI3K-activated pathway. To support our evidence linking PI3K to S6K in Axl signalling, we analyzed the effect of wortmannin on Gas6-dependent activation of S6K. Quiescent NIH 3T3 cells were treated for 30 min with 1 μM wortmannin before being stimulated with Gas6 (400 ng/ml) or bFGF (100 ng/ml), and S6K activation was evaluated as described above. Drug addition completely blocked S6K activation as induced by Gas6 while having no effect on bFGF used as control (Fig. 5A). Our results are consistent with the data reported for PDGF and insulin (10, 13), thus indicating PI3K as a mediator in Gas6-dependent activation of S6K in serum-starved cells.

Akt kinase is a downstream target of PI3K that is inactive in serum-starved NIH 3T3 cells and is rapidly activated by growth factor addition (21). Involvement of the PI3K-stimulated pathway was therefore investigated by analyzing the activation of Akt. Serum-starved NIH 3T3 cells were stimulated for 10 min with Gas6, and cell lysates were analyzed for Akt activation. The direct activation of Akt by the receptor was analyzed by treating separate petri dishes with wortmannin or rapamycin

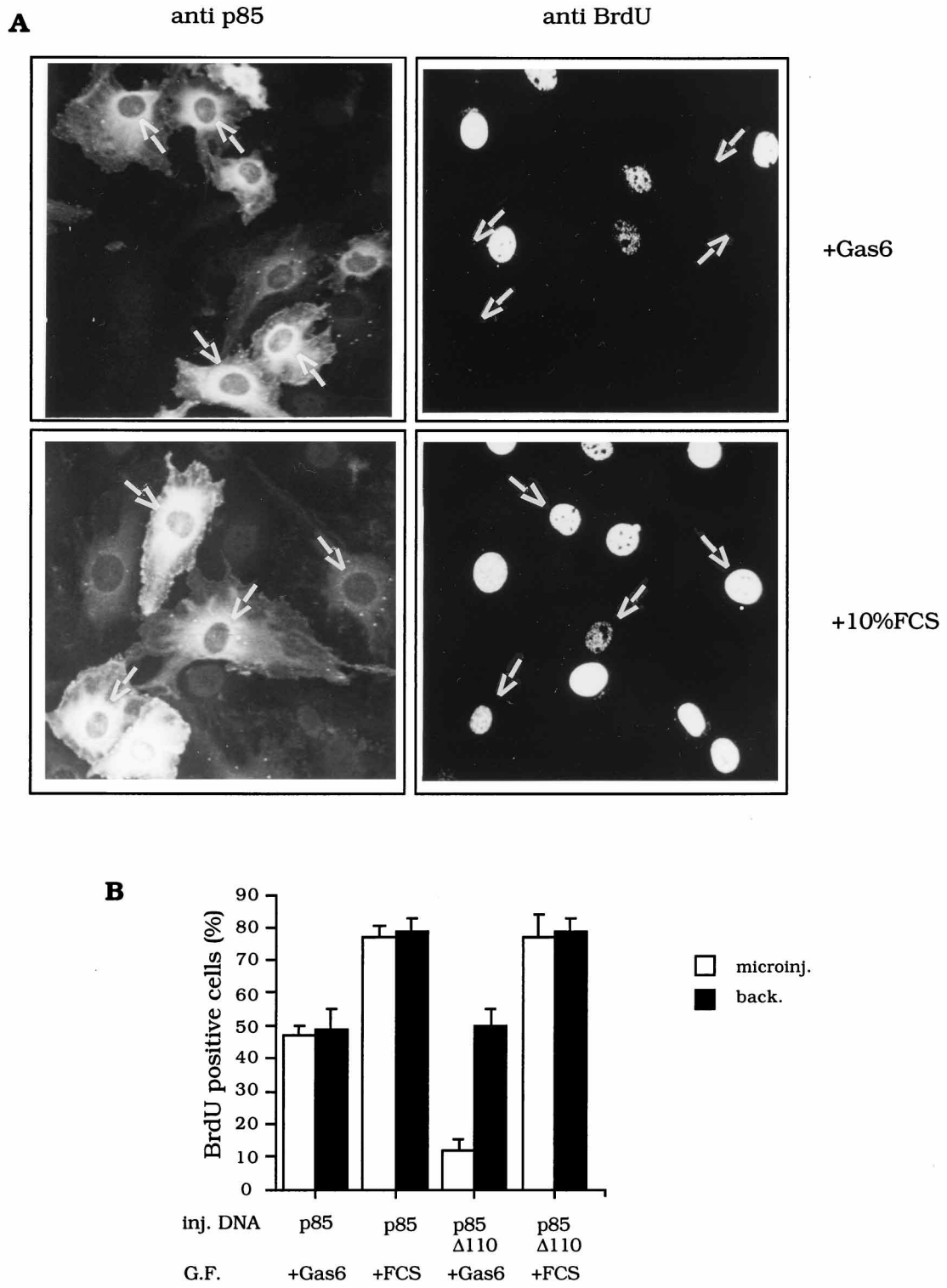

FIG. 3. Dominant negative p85 subunit blocks Gas6-induced mitogenesis. Growth-arrested NIH 3T3 cells were given microinjections of pGDVS7-p85Δ110 cDNA (20 ng/μl) encoding the dominant negative p85 deletion mutant, and after 4 h the cells were stimulated either with 400 ng of Gas6 per ml (+Gas6) or 10% FCS (+10FCS). BrdU was added together with the growth factors to monitor entry into the S phase. As a control, NIH 3T3 cells were given microinjections of wild-type pGDSV7-p85 cDNA (20 ng/μl) and similarly stimulated. After the indicated times, the cells were fixed and double immunofluorescence was performed with anti-p85 and anti-BrdU monoclonal antibodies. The second antibodies were goat anti-IgG1 FITC (Southern) for p85 (anti-p85) (17) and goat anti-IgG2a RITC (Southern) for BrdU (anti-BrdU). (A) Representative field of cells given microinjections of pGDSV7-p85Δ110. Arrows indicate the positions of the nuclei (anti-BrdU) of p85Δ110-overexpressing cells (anti-p85) stimulated with Gas6 (+Gas6) or FCS (+10%FCS). (B) Effect of p85 and p85Δ110 microinjection on Gas6-induced entry into the S phase. The ordinate indicates the percentage of cells given microinjections of wild-type p85 (p85) or dominant negative p85 (p85Δ100) and noninjected background cells incorporating BrdU after Gas6 or FCS addition. Error bars above the columns represent the standard deviation. More than 300 injected cells were analyzed, and the results of four independent experiments are reported here. Immunofluorescence analysis was performed with an Axiovert 35 microscope (Zeiss), and images were obtained with a confocal laser scan microscope (Zeiss).

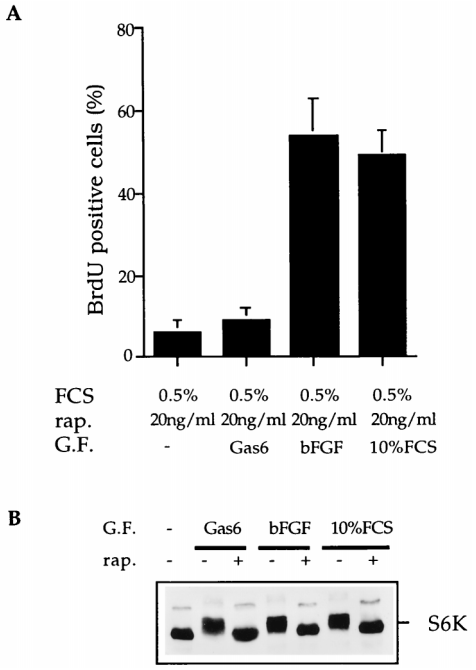

FIG. 4. Rapamycin inhibits Gas6-induced mitogenic activity. (A) Analysis of S-phase induction in the presence of rapamycin. Serum-starved NIH 3T3 cells were pretreated with rapamycin (20 ng/ml) for 20 min before the addition of either Gas6 (400 ng/ml), bFGF (100 ng/ml), or 10% FCS. The mitogenic assay was carried out as described in the legend to Fig. 1A. The results of four separate experiments are shown; the bar above the histograms represents the standard deviation from the mean. (B) S6K activation after Gas6 addition. Growth-arrested NIH 3T3 cells were treated (+) or not treated (−) with rapamycin (rap.) (20 ng/ml) and stimulated for 10 min with Gas6, bFGF, or 10% FCS. Western blot analysis was performed on total-cell lysates with a polyclonal anti-S6K antibody. Activation and phosphorylation of S6K correlate with the appearance of slower-migrating species on SDS-PAGE.

before stimulation as previously reported (21). Figure 5B shows the results of the Western blot analysis of total-cell lysates before and after stimulation with Gas6. Akt phosphorylation, as assessed by the appearance of the electrophoretic lower-migrating form, was evident only after Gas6 addition; this activation was abolished by wortmannin treatment. Consistent with previous reports (6, 21), rapamycin addition had no significant effects on Akt activation. Therefore, activation of the PI3K pathway and Akt by Gas6 appear to be functionally linked, thus showing relevant similarities to the PDGF-activated pathway.

Gas6 stimulation activates c-Src. Stimulation of quiescent fibroblasts by PDGF, EGF, and insulin causes a transient increase in the intrinsic activity of the Src tyrosine kinases (18, 64). We therefore analyzed the ability of Axl to activate c-Src after addition of Gas6 to serum-starved cells. NIH 3T3 cells were serum starved in a 15-cm petri dish and stimulated for 0, 10, 20, and 30 min with 400 ng of recombinant Gas6 per ml. After this time, the cells were washed with cold PBS and Src proteins were immunoprecipitated essentially as described previously (37). Equal amounts of total lysates were incubated for 1 h on ice with an anti-Src polyclonal antibody (67). The immunocomplexes were equilibrated in kinase buffer and incubated for 20 min at 30°C in the presence of [γ-^{32}P]ATP to monitor kinase activation. The reactions were stopped by adding SDS-PAGE loading buffer for subsequent SDS-PAGE and autoradiography. Figure 6A shows the result of a typical experiment indicating that a significant increase in autophosphorylation of endogenous c-Src was observed after Gas6 addition. Activation was transient, reaching a peak 10 min after addition of Gas6 to serum-starved cells as similarly described for other growth factors (18).

Src activation is accompanied by novel phosphorylations on serine and tyrosine residues (27). We therefore analyzed whether Gas6 activation of Src could be indicated by using anti-phosphotyrosine antibodies. COS-7 cells were transfected with pSG5 c-src K+ expression vector, and 24 h after transfection the COS-7 cells were serum starved for 24 h (0.5% FCS). A separate petri dish was mock transfected and used as control. After this time, the cells were stimulated for 10 min with Gas6 (400 ng/ml, final concentration) and lysed by adding SDS loading buffer directly to the petri dishes. Equal amounts of proteins were loaded on SDS-PAGE gels (10% polyacrylamide), and after immunoblotting, the membrane was revealed with anti-phosphotyrosine monoclonal antibodies (PY20). As shown in Fig. 6B, Gas6 treatment of transfected cells resulted in a significant increase in Src phosphotyrosine content, thus confirming the ability of Gas6 to activate both the endogenous and ectopically overexpressed c-Src.

Src is required for Gas6-induced mitogenesis. Src kinases are critically involved in different growth factor receptor pathways (61, 64). Microinjection of antibodies specific for the Src family kinases Src, Fyn, and Yes (54) or of cDNA expressing a catalytically inactive *src* or *fyn* (62) was used to demonstrate the requirement of Src family for several growth factor receptors. We therefore tested whether Src is similarly required for Gas6 induced mitogenesis. Quiescent NIH 3T3 cells were given a microinjection of an expression plasmid coding for a kinase-inactive form of Src (Src K−) (kindly provided by S. Courtneidge). At 4 h later, S-phase reentry was induced in

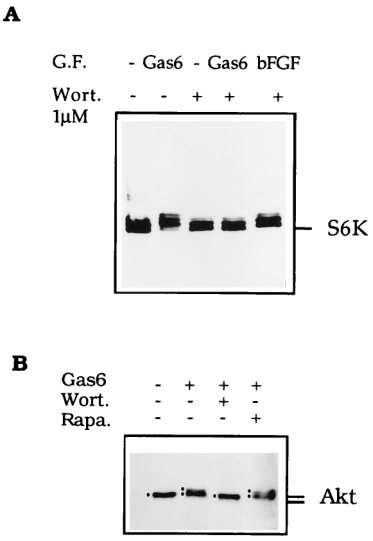

FIG. 5. Gas6 activates downstream elements in the PI3K pathway. (A) Wortmannin addition prevents S6K activation. Growth-arrested NIH 3T3 cells were treated (+) or not treated (−) with 1 μM wortmannin (Wort.) before Gas6 addition (400 ng/ml, final concentration). A parallel experiment was carried out with bFGF (100 ng/ml) as a control. Western blot analysis of S6K activation was performed as described in the legend to Fig. 4B. (B) Gas6 induces phosphorylation of Akt kinase. Western blot analysis with anti-Akt antibodies was performed on cellular lysates from serum-starved NIH 3T3 cells stimulated (+) or not stimulated (−) for 10 min with Gas6 (400 ng/ml, final concentration). Activation of Akt results in phosphorylation and the appearance of a slower-migrating form on SDS-PAGE (indicated by dots). In separate experiments, cells were pretreated with either 1 μM wortmannin (Wort.) or 20 ng of rapamycin (Rapa.) per ml before Gas6 addition.

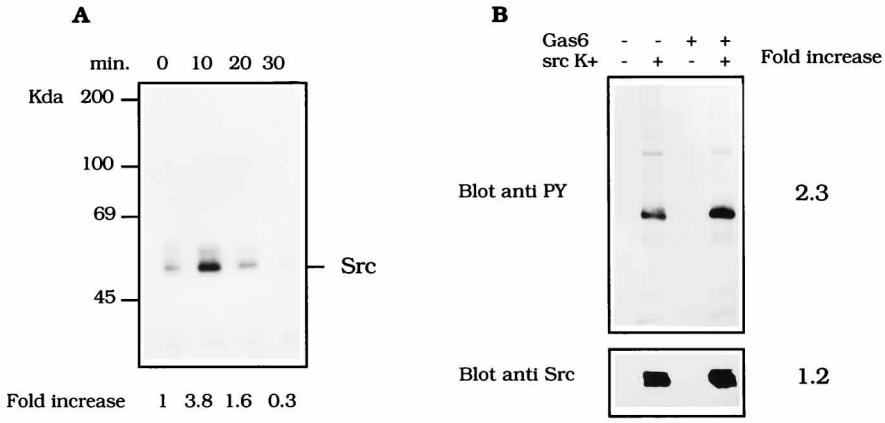

FIG. 6. Gas6 addition to serum-starved cells activates c-Src. (A) Activation of Src kinase after Gas6 addition. A kinase assay was performed with serum-starved NIH 3T3 cells stimulated with 400 ng of Gas6 per ml for 0, 10, 20, or 30 min. Equal amounts of total-cell lysates were immunoprecipitated with anti-Src polyclonal antibodies (67), and the kinase assay was performed as indicated in Materials and Methods. The autoradiography is representative of three separate experiments. (B) Gas6 induces tyrosine phosphorylation of Src. Western blot analysis with anti-phosphotyrosine antibodies on cellular lysates from COS-7 cells transfected (+) or not transfected (−) with pSG5 wild-type c-Src (src K+), and treated (+) or not treated (−) with Gas6 (400 ng/ml) for 10 min (upper panel). Equal amounts of loaded proteins were assessed with separate Coomassie stained gels. A separate Western blot analysis was carried out with monoclonal anti-Src antibodies (327Ab [41]) as controls (lower panel).

separate experiments by the addition of Gas6, PDGF, bFGF, or FCS and followed by BrdU incorporation. After 16 h, cells were fixed and processed for double immunofluorescence with anti-Src (41) and anti-BrdU antibodies. As shown in the representative field of Fig. 7A and the histograms of Fig. 7B, overexpression of the dominant negative Src K− strongly inhibited BrdU incorporation induced by Gas6 (7.5% ± 5.5% cells in S phase compared to 35% ± 3% cells in S phase in the background noninjected cells). This effect was similar to that previously described by Twamley-Stein et al. (62), for PDGF, here used as a positive control in the experiment (20% ± 3% cells in S phase compared to 57% ± 4.7% cells in S phase in the background). No significant effect was observed when cells were stimulated with either bFGF (50.7% ± 0.9% compared to 53.7% ± 1.2% cells) or 10% FCS (53% ± 4.9% compared to 61.7% ± 5.4%). Therefore, Src K− expression specifically interfered with Gas6 mitogenic pathway, as shown for PDGF (Fig. 7A and B). No inhibitory effect on Gas6 mitogenesis was detected when Src K+ was microinjected in separate experiments (not shown).

To substantiate these findings, stable transfectants expressing the same dominant negative Src were obtained from NIH 3T3 fibroblasts. Asynchronously growing cells were transfected simultaneously with pSG5 Src K− DNA together with neomycin resistance. Clones were selected for G418 resistance and screened for Src expression by Western blotting with anti-Src antibodies. Figure 8 shows the Western blot analysis of total-cell lysates from various isolated NIH 3T3 clones (SR) expressing different relative levels of Src K−, with respect to a neomycin-resistant clone used as a control. Equal amounts of proteins were adjusted relative to the expression of Axl receptor in a separate Western blot (Fig. 8A). The clones with the highest (SR4) and lowest (SR5) levels of Src K− expression were selected for further analysis. SR5 behaved in all the assays similarly to the neomycin-resistant control (data not shown). No significant effect on cell growth was found for Src K−, as assessed by analysis of BrdU incorporation into asynchronously growing cells. Moreover, the various clones showed normal growth arrest features after serum starvation. Consistent with the results obtained by microinjection, NIH 3T3 stably overexpressing the dominant negative Src (SR4) failed to enter the S phase after stimulation with Gas6 (400 ng/ml, final concentration), in contrast to the control SR5, which efficiently responded to Gas6 (Fig. 8B). Both SR4 and SR5 clones previously arrested by serum deprivation synchronously reentered the S phase after 10% FCS addition, with similar kinetics to those of untransfected NIH 3T3 cells (data not shown). The defective mitogenic response to Gas6 was not a peculiarity of the SR4 clone: in fact, SR1 and SR3 also failed to enter the S phase after Gas6 stimulation (not shown). Together, the results obtained in both transient- and stable-expression assays confirmed the requirement of Src or something similar for the mitogenic effect of Gas6.

Gas6 regulated survival pathway. We previously reported that Gas6 is able to efficiently prevent apoptosis induced by complete serum withdrawal from serum-starved NIH 3T3 cells. This survival activity was shown to be uncoupled from mitogenesis since Gas6 is not able to induce BrdU incorporation in the complete absence of serum (26).

To investigate whether wortmannin and rapamycin were able to interfere with Gas6-induced survival, the proteolytic cleavage of Gas2, a substrate for ICE-like protease, was analyzed (4). Serum-starved NIH 3T3 cells were pretreated by adding either 1 μM wortmannin or 20 ng of rapamycin per ml to the starvation medium. Apoptosis was then induced by changing the medium to serum-free medium containing freshly added wortmannin or rapamycin. To analyze the effect of wortmannin and rapamycin on Gas6 survival, separate petri dishes were treated under these conditions with Gas6 (400 ng/ml) or bFGF (100 ng/ml) as a control. Both adherent and nonadherent apoptotic cells present in the medium were combined for Western blot analysis with anti-Gas2 antibodies. Figure 9A shows that both wortmannin and rapamycin completely abolished the ability of Gas6 to prevent Gas2 apoptosis-associated proteolytic cleavage. Consistent with the results obtained in the mitogenic assays, bFGF survival activity was not affected by the addition of drugs, as can be seen by the lack of Gas2 processing (Fig. 9A). Although Gas6 mitogenic and survival activities could be uncoupled, these results indicate that the components involved in Gas6 induced cell cycle reentry are shared with its survival activity.

Similarly, we investigated whether Gas6-induced survival was also dependent on Src. The SR4 cell line was serum starved for 48 h, and apoptosis was induced as above by com-

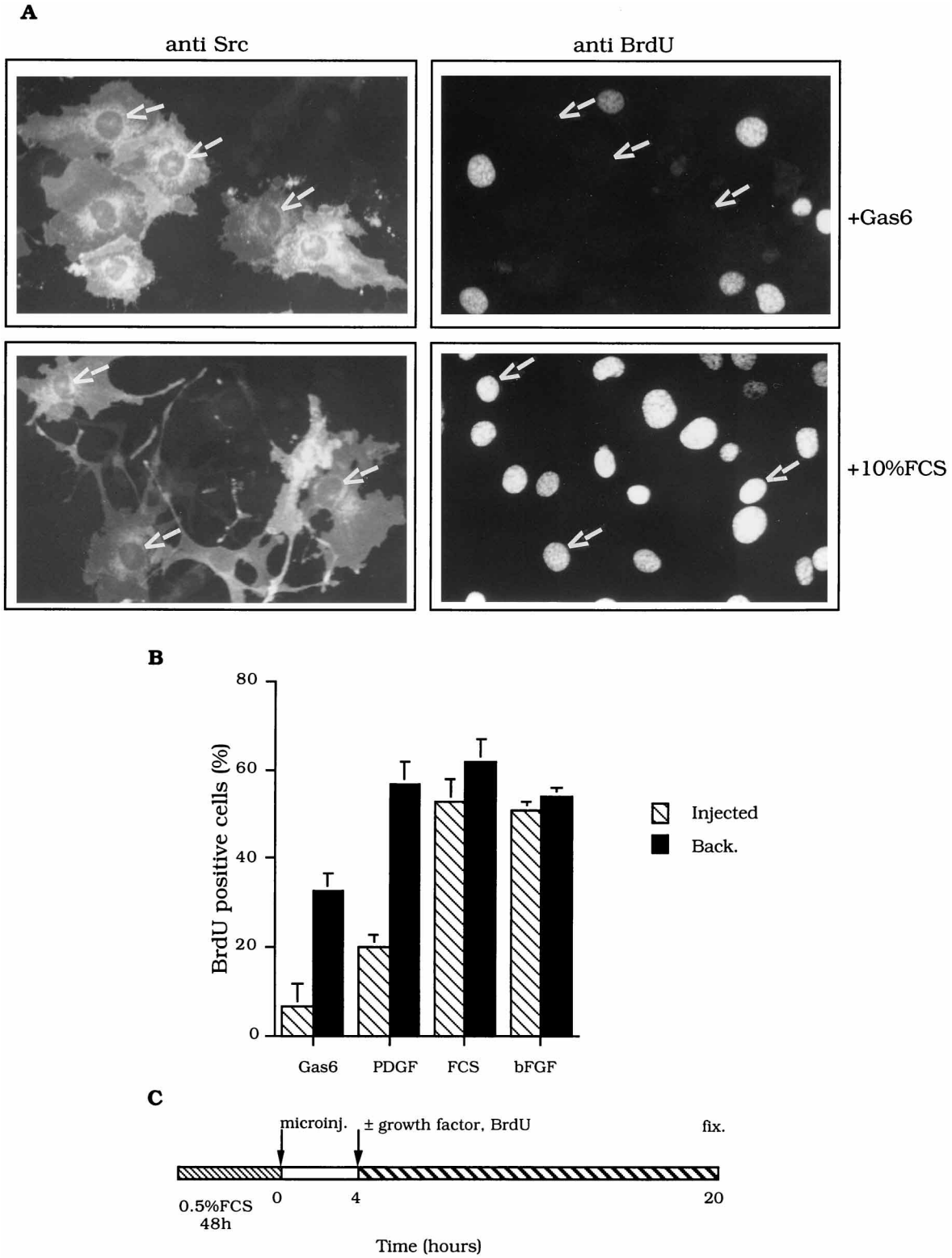

FIG. 7. Transient expression of Src K− inhibits Gas6-induced DNA synthesis. Growth-arrested NIH 3T3 cells were given microinjections of an expression plasmid (pSG5-src K−; 10 ng/μl) encoding catalytically inactive c-Src. At 4 h later, Gas6, PDGF, bFGF, and 10% FCS were added together with BrdU (50 μM) for a further 16 h. Double immunofluorescence was carried out with monoclonal antibodies anti-Src (327Ab) and anti-BrdU. Second antibodies were goat anti-IgG1 FITC (Southern) for Src (anti-Src) and goat anti-IgG2a RITC (Southern) for BrdU (anti-BrdU). (A) Microinjection fields of a representative experiment. Arrows indicate Src K− overexpressing cells (anti Src) and the correspondent staining with anti-BrdU (anti BrdU) of cells stimulated with either Gas6 (+Gas6) or FCS (+10%FCS). Images were obtained with a laser scan microscope (Zeiss). (B) Effect of Src K− expression on Gas6-, PDGF-, bFGF-, and FCS-induced entry into the S phase. The ordinates indicate the percentage of cells incorporating BrdU positive for Src K− and the relative uninjected background calculated as reported in the legend to Fig. 1A. The mean of four independent experiments and the standard deviation of the mean are summarized. More than 300 microinjected cells were analyzed in each experiment. (C) Scheme of the reported experimental protocol as shown in panels A and B.

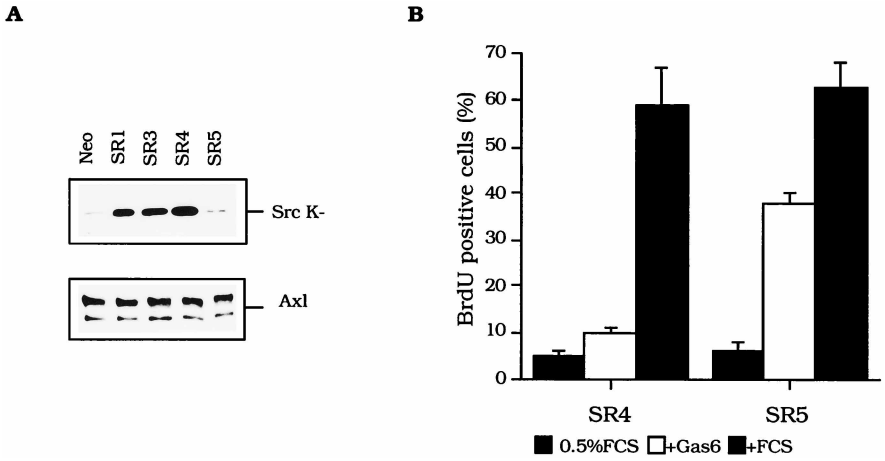

FIG. 8. Src K− stably-expressing NIH 3T3 cells are refractory to Gas6 mitogenic effect. (A) Analysis of Src K− expression in stable cell lines. NIH 3T3 cells were cotransfected with pSG5 Src K− and a plasmid carrying the neomycin phosphotrasferase cDNA. Relative levels of Src K− expression were assessed by Western blot analysis with anti-Src monoclonal antibodies (327Ab) from lysates of the various obtained clones (SR1, SR3, SR4, and SR5). A clone transfected only with the neomycin resistance gene (Neo) was used as a negative control. Equal protein amounts from each clone were assessed on a separate Coomassie-stained gel and by Western blotting with anti-Axl polyclonal antibodies (lower panel). (B) Analysis of the mitogenic activity of Gas6 in two NIH 3T3 clones. Two separate clones, SR4 and SR5, expressing different levels of Src K− as assessed in panel A were used. The clones were serum starved for 48 h and either left untreated or stimulated with 400 ng of Gas6 per ml or 10% FCS. The mitogenic assay was carried out as described in the legend to Fig. 1. The mean of four independent experiments and the standard deviation from the mean are shown.

plete serum removal. Total-cell lysates of adherent and nonadherent cells were combined and analyzed by Western blotting for Gas2 cleavage (Fig. 9B). Gas2 protein integrity on adherent serum-starved cells at the beginning of the experiment was also analyzed (Fig. 9B, lane A). Gas6 was not able to block apoptosis in the SR4 clone, whereas bFGF efficiently prevented cell death (Fig. 9B), thus suggesting that Src was also involved in Gas6 antiapoptotic signalling. Such involvement was further investigated by transient-expression assays by microinjection of Src K− in serum-starved NIH 3T3 cells. Cells seeded on a coverslip were microinjected with the expression vector encoding the kinase-inactive c-Src (Src K−), and after 4 h the starvation medium was changed to serum-free medium to induce apoptosis. The total number of injected cells

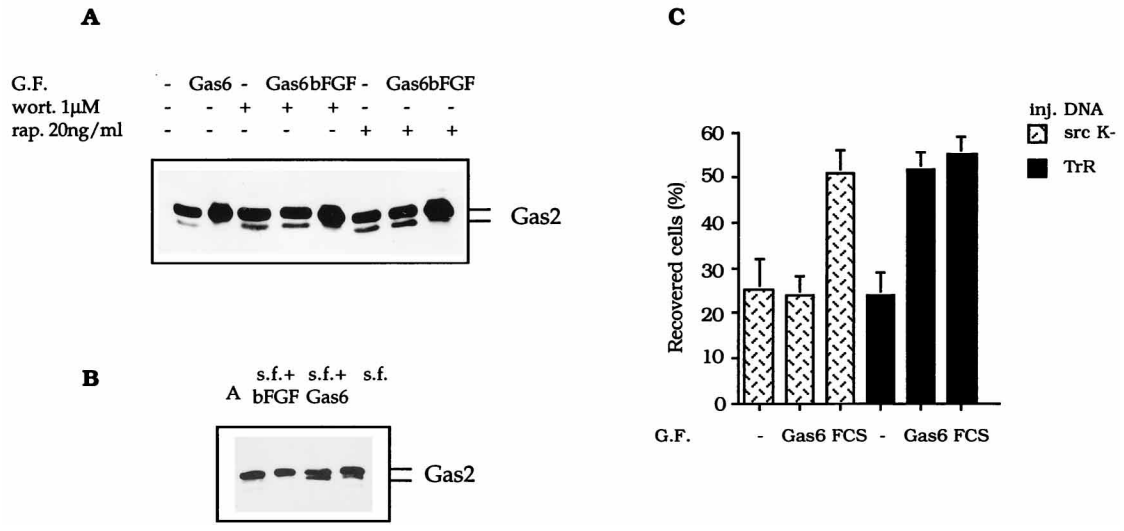

FIG. 9. Gas6 survival pathway. (A) Wortmannin and rapamycin block Gas6 antiapoptotic activity. Western blot analysis with anti-Gas2 antibody on cellular lysates derived from serum-starved NIH 3T3 cells incubated for 24 h in serum-free medium (−) or serum-free medium supplemented with Gas6 (+Gas6) or bFGF (+bFGF). Separate petri dishes were treated with wortmannin (1 μM) or rapamycin (20 ng/ml) and similarly stimulated with Gas6 or bFGF. For Western blot analysis, both adherent and nonadherent cells were combined; Gas2 cleavage becomes evident as a higher-mobility form in SDS-PAGE (4). (B) Lack of Gas6-dependent survival in NIH 3T3 cells stably expressing the dominant negative Src. Western blot analysis with anti-Gas2 antibody on cellular lysates derived from adherent serum-starved GSR4 cells (lane A) and after 24 h of incubation in serum-free medium (S.F) or serum-free medium supplemented with bFGF (S.F.+bFGF) or Gas6 (S.F.+Gas6 is shown). For Western blot analysis of cells in serum-free medium, adherent and nonadherent cells were combined and Gas2 cleavage was evidenced as in panel A. (C) Transient overexpression of Src K− impairs Gas6 survival activity. Serum-starved NIH 3T3 cells were given microinjections of the plasmid carrying the dominant negative Src (Src K−) as in Fig. 5. After 4 h, apoptosis was induced by complete serum withdrawal from the medium, and the cells were incubated for a further 16 h. Gas6 or FCS was added to the medium as survival factors. After this time, the cells were analyzed by immunofluorescence with anti-Src antibody (327Ab). A parallel experiment was carried out with TrR under the same conditions as a control. The immunofluorescence was done with monoclonal anti TrR antibody (OKT9) and anti-IgG1 FITC as the second antibody (Southern). The mean of four independent experiments and the standard deviation of the mean are shown.

were counted during the microinjection time and after immunofluorescent staining for Src overexpression. Survival was expressed as the percentage of cells stained for c-Src that were recovered. As a control, the cDNA encoding the human transferrin receptor (TrR) was similarly expressed by microinjection and cells similarly scored for TrR expression. Figure 9C shows that expression of the dominant negative Src abolished Gas6-induced survival while not interfering with FCS-induced survival. Conversely, when TrR cDNA was similarly microinjected, Gas6 was able to recover the TrR-overexpressing cells as efficiently as FCS under similar conditions (Fig. 9C). These results confirm our previous findings (26) that Gas6 is able to recover cell death as induced by complete serum withdrawal and show that antiapoptotic activity absolutely requires the presence of an active Src kinase.

DISCUSSION

A large body of evidence indicates that one function of growth factors besides their mitogenic activity is to suppress apoptosis (30, 31, 52). As suggested, restriction of the apoptotic program should be an obligatory component for cell cycle reentry (19). In this light, the signals provided by growth factors to suppress apoptosis become a prerequisite for their mitogenic potential. In fact, insulin-like growth factor (IGF) and PDGF were reported to suppress apoptosis in fibroblasts and postmitotic neurons under conditions where cell proliferation was blocked (2, 18). Consistently, we have shown that Gas6 prevents cell death in the complete absence of growth factors without exerting a mitogenic effect (26). Since Gas6 expression is enhanced during growth arrest (58), such survival activity is likely to be relevant for quiescent cells. Nevertheless, we used the reported mitogenic activity that Gas6 exerts in the presence of the limiting serum amount present in the starvation medium (26, 39) as a tool to gain further insights into Gas6-activated signalling for NIH 3T3 cells. Two potential p85 binding sites identified in the Axl cytoplasmic domain prompted us to investigate the involvement of PI3K in Gas6-induced mitogenesis. By using the PI3K inhibitor wortmannin and a dominant negative p85, which is unable to bind the p110 catalytic subunit (p85Δ110), we showed a requirement for PI3K in Gas6 signalling. Consistently, Fridell et al. have demonstrated, by using a 32D cell system and a series of Axl mutants, that Gas6 induces the binding of the p85 component to Axl (22). Although a clear association of PI3K was observed, no mitogenic or antiapoptotic activity was observed and PI3K appeared to be dispensable for receptor activity (22). A possible explanation for such discrepancies with our results could be found considering the expression of Axl-related receptors. On this line and as demonstrated for the EGF family of RTK (9), Axl may heterodimerize with other members of this RTK family and generate different subsets of tyrosines for signalling. In fact, unlike 32D cells, NIH 3T3 cells also express the Axl-related Sky/Rse receptors, which we found capable of heterodimerizing with Axl following Gas6 addition to serum-starved cells (24a).

Gas6 activation of known downstream molecular targets for PI3K, S6K and Akt, was demonstrated. Rapamycin inhibitor abolished Gas6-induced mitogenesis, confirming the requirement of S6K for Gas6 signalling. (Involvement of the PI3K activated pathway was not confined to NIH 3T3 cells but was a more general feature, since we could obtain similar results with human WI38 fibroblasts [data not shown].) Given an analogous requirement for PI3K as reported for PDGF (54), we analyzed whether Src was also involved in Gas6 signalling. Even though the Axl cytoplasmic region lacks any known Src-interacting consensus sequence (47) and Src could not be coimmunoprecipitated with the Axl receptor (data not shown), Gas6 addition stimulated the activation of endogenous Src. Consistently, transient expression by microinjection of a dominant negative kinase-inactive c-Src (Src K−) abolished Gas6 mitogenic effect in serum-starved NIH 3T3 cells. Moreover, NIH 3T3 cells stably expressing the same dominant negative Src were unresponsive to the Gas6 mitogenic effect, thus supporting Src involvement in Gas6 signalling. These results are reminiscent of the reported EGF-induced 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 (55).

We have previously reported evidence showing that Gas6 efficiently protects serum-starved NIH 3T3 cells from apoptosis by complete growth factor withdrawal (26). Apoptosis is characterized by the activation of specific proteolytic pathways, and these were shown to be efficiently blocked by Gas6 addition (26). By using Gas2 cleavage (4) as a marker for apoptosis, both wortmannin and rapamycin abrogated Gas6-dependent survival activity, thus indicating that a similar pathway is activated when Gas6 exerted its antiapoptotic effect. Accordingly, transient expression of a dominant negative Src dramatically decreased the cell recovery as induced by Gas6 addition as well as stable expression of the same dominant negative Src.

Our results raise the question of how Src and PI3K are connected in Gas6 signalling. A number of reports have described that the SH3 domains of v-Src, Fyn, Lyn, and Lck are capable of interacting with p85 (50, 64). Since Axl does not present binding sites for Src, an SH3-mediated interaction of Src with p85 could be responsible for regulating elements in the PI3K pathway that are required for Axl. In this light, the effects of Src K− should be explained as forming inactive complexes with p85, thus preventing such function. An alternative hypothesis considers that PI3K and Src supply two separate signals: Src should activate a pathway leading to expression of Myc (1), while the PI3K pathway might provide the survival inputs required to overcome apoptosis induced by Myc expression in serum-starved cells (30). Along these lines, the PI3K-activated pathway was recently demonstrated to be involved in the regulation of cell survival through the kinase Akt/PKB (20), and the latter was shown to prevent Myc-induced cell death (34).

Since Gas6 in the complete absence of serum does not promote S-phase entry, it is highly unlikely that Myc expression should be the primary target of Src under these conditions. Therefore, the role of Src should be to potentiate the effects on the PI3K survival pathway in line with the first hypothesis, thus participating in the control of Gas6 over cell death (46). Finally, since both Gas6 mitogenesis and survival seem to require activation of similar elements, the choice between these two responses may bifurcate downstream S6K and be dependent on other growth factors contained in the low serum concentration that is present in the starvation medium.

In summary, our results showed a requirement for PI3K, S6K, and Src for the mitogenic effect of Gas6. This investigation allowed us to uncover the role of these elements in Gas6-induced survival and for the first time to identify a role for a member of the Src tyrosine kinase family in the control of apoptosis in NIH 3T3 fibroblasts.

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