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Differential Activation of the Ras/Extracellular-Signal-Regulated Protein Kinase Pathway Is Responsible for the Biological Consequences Induced by the Axl Receptor Tyrosine Kinase

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To understand the mechanism of Axl signaling, we have initiated studies to delineate downstream components in interleukin-3-dependent 32D cells by using a chimeric receptor containing the recombinant epidermal growth factor (EGF) receptor extracellular and transmembrane domains and the Axl kinase domain (EAK [for EGF receptor-Axl kinase]). We have previously shown that upon exogenous EGF stimulation, 32D-EAK cells are capable of proliferation in the absence of interleukin-3. With this system, we determined that EAK-induced cell survival and mitogenesis are dependent upon the Ras/extracellular-signal-regulated protein kinase (ERK) cascade. Although the phosphatidylinositol-3 kinase pathway is activated upon EAK signaling, it appears to be dispensable for the biological actions of the Axl kinase. Furthermore, we demonstrated that different threshold levels of Ras/ERK activation are needed to induce a block to apoptosis or proliferation in 32D cells. Recently, we have identified an Axl ligand, GAS6. Surprisingly, GAS6-stimulated 32D-Axl cells exhibited no blockage to apoptosis or mitogenic response which is correlated with the absence of Ras/ERK activation. Taken together, these data suggest that different extracellular domains dramatically alter the intracellular response of the Axl kinase. Furthermore, our data suggest that the GAS6-Axl interaction does not induce mitogenesis and that its exact role remains to be determined.

The transforming gene *axl* encodes a novel receptor possessing tyrosine kinase activity and was first isolated from the DNA of patients with chronic myelogenous leukemia (18, 33). The transforming activity of *axl* was first identified by a sensitive nude mouse tumorigenicity assay. The *axl* gene product displays homology to several known tyrosine kinases and yet contains unique sequences in the kinase domain (KWIAIE) (33). The unique positioning of immunoglobulin-like and fibronectin III repeats in the extracellular region makes Axl a structurally novel RTK (receptor tyrosine kinase) with features resembling those of cellular adhesion molecules such as the neuronal cell adhesion molecule, fasciculin, and receptor tyrosine phosphatases (33). Recently, a growing number of Axl-related RTKs which are expressed predominantly in the central nervous system have been identified (23, 26, 34). Axl, however, is expressed in developing mesenchymal tissues after day 7 of murine development. In adult mice, the Axl protein is found in many tissues including the heart, gonads, Purkinje cells of the cerebellum, and kidneys (13a). Furthermore, our preliminary data suggest that overexpression of Axl by using a β-actin promoter in transgenic mice appears to cause prenatal lethality (8a). Taken together, these results suggest that Axl plays a role in normal development. It has been recently demonstrated that the mouse homolog of Axl, adhesion-related kinase ARK, is able to induce cell aggregation via homophilic association of the extracellular domain of ARK (2). Moreover, this homophilic binding induces receptor phosphorylation. Thus, it is possible that this unique mechanism is employed to activate this group of RTKs.

To understand the mechanism of Axl-mediated signal transduction, much effort has been focused on the identification of its ligand. Recently, an Axl ligand, GAS6 (encoded by a growth arrest-specific gene), has been identified (52). GAS6 was previously identified and cloned by virtue of its up-regulated expression upon serum starvation in NIH 3T3 cells (25). In addition, it has been demonstrated that GAS6 is also a ligand for the Axl-related RTK rse/Tyro3 (12). Sequence analysis revealed that GAS6 has 44% identity with protein S, a natural anticoagulation factor. The function of GAS6 activation of Axl/Tyro3 remains unclear. The mitogenic action of GAS6 in Axl-expressing cells is, at best, weak (52). It has recently been proposed that one biological function of GAS6 may be to potentiate cell proliferation in vascular smooth muscle cells mediated by Ca^{2+}-mobilizing receptors (31). These findings suggest that unlike "classical" growth factor receptors, Axl and Axl-related receptors may signal through mechanisms that result in distinct cellular consequences.

Prior to identification of the Axl ligand GAS6, we initiated studies of Axl signaling in interleukin-3 (IL-3)-dependent cell line 32D by using a chimeric receptor containing the epidermal growth factor (EGF) receptor (EGFR) extracellular and transmembrane domains and the Axl kinase domain (EAK [for EGFR-Axl kinase]). We have previously shown that upon ex-

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ogenous EGF stimulation, 32D-EAK cells are capable of proliferation in the absence of IL-3 (29). While normal growth of 32D cells is absolutely dependent upon IL-3, prolonged activation of EAK renders these cells permanently independent of IL-3 or EGF for growth. This factor independence correlates with overexpression of the Axl receptor. These biological responses are associated with phosphorylation of EAK on tyrosine residues, indicating that activation of the Axl kinase can induce growth signals in a myeloid cell context.

Herein, we present results demonstrating that EAK signals through the Ras/extracellular-signal-regulated protein kinase (ERK) pathway. A panel of EAK mutants and inhibitors of signaling molecules have enabled us to dissect distinct EAK-mediated biological outcomes as a result of differential activation of the Ras/ERK pathway. Intriguingly, although we have demonstrated that the p85 subunit of the phosphatidylinositol-3 (PI-3) kinase is associated with the activated Axl kinase and that the PI-3 kinase activity associated with the receptor increased moderately upon receptor activation, no measurable biological consequence of this association was detected. Therefore, the role of the PI-3 kinase pathway in Axl signaling appears to be dispensable. Furthermore, the biochemical characterization of GAS6-Axl interactions has revealed that unlike the EGFR-Axl chimeric receptor, the Ras/ERK pathway is not involved in GAS6-induced Axl signaling. The biologically different outcomes resulting from EAK signaling compared with GAS6-Axl interactions suggest that the configuration of the extracellular domain has a dramatic impact on the downstream signaling events induced by the intracellular kinase of Axl.

MATERIALS AND METHODS

Generation of mutant EAK constructions. The wild-type EAK construction was made as described previously (29). Specific mutations in the Axl kinase domain of EAK were generated with the Altered Sites in vitro mutagenesis system (Promega). For mutagenesis, the wild-type Axl intracellular domain was subcloned into the *Sal*I and *Bam*HI sites of the pALTER-1 vector. The primers for the mutant constructions are as follows (mutated sequences are underlined; deleted sequences are in brackets): EAK567R, ATCTTCATCGTCCGCAC AGCCACCTT;EAKM824A, 5′-TCCACCCTCATCTGCGTTGACATAGAGG ATTTCGTC-3′; EAKΔPI-1, 5′-CCAGCACCGCGA[CATCAAGGCATA]CA GTCCATCCAG-3′; EAKΔPI-2, 5′-TCCACCCTCATC[CATGTTGACATA] GAGGATTTCGTC-3′. Amino acids 779 to 782 were deleted in the EAKΔPI-1 mutant, whereas amino acids 821 to 824 were deleted in the EAKΔPI-2 mutant. The double mutant construction EAKΔPI was made by using the primers for both EAKΔPI-1 and EAKΔPI-2. Following in vitro mutagenesis and sequencing, the Axl intracellular domain was subcloned into the *Sal*I and *Sma*I sites of the pBluescript vector containing the recombinant EGFR (rEGFR) extracellular and transmembrane domains (29). The entire mutant EAK construction was then subcloned into the *Hpa*I and *Xho*I sites of mammalian expression vector pLXSN (29).

To generate dominant negative ERK mutants under the control of the dexamethasone-inducible mouse mammary tumor virus promoter, the coding region of the hemagglutinin (HA)-tagged ERK52R and ERK71R constructions was subcloned into the *Not*I and *Eco*RI sites of the pMAM expression vector (Clontech). The HA-tagged ERK mutants were kindly provided by M. Cobb. (University of Texas Southwestern Medical Center) (41).

Cell lines, ligand stimulation, and retroviral infections. Murine IL-3-dependent 32D C13 (32D) cells and the murine retrovirus packaging cell line GP+E86 were maintained as previously described (27, 29). The EAK receptor was activated with human rEGF (100 ng/ml) (GIBCO-BRL). The full-length Axl receptor was activated with human recombinant GAS6 (100 ng/ml) (provided by B. Varnum, Amgen). Platelet-derived growth factor B chain (PDGF-BB), at a concentration of 2 ng/ml (Genzyme), was added to activate the human PDGF receptor (PDGFR) in 32D cells (gift of J. Pierce, National Institutes of Health). For ligand treatment, 10^7 cells were serum starved for 4 h in RPMI 1640 at 37°C. Cell were then treated with the appropriate ligand for 10 min at 37°C.

The GP+E86 cell line was used to generate recombinant retroviruses containing various EAK mutant constructs as previously described (29). In brief, the EAK mutant constructions were transfected with lipofectin (GIBCO-BRL) into GP+E86 cells. G418-resistant colonies were selected and used to prepare virus stocks (27). For infection, 10^6 32D cells were incubated with recombinant retrovirus (10^5 CFU/ml) for 24 h in medium containing IL-3. Infected 32D cells were then selected in medium containing IL-3 and G418 (800 μg/ml). Polyclonal populations were expanded and maintained in the presence of G418.

Transfection of dominant negative ERK mutants. The pMAM, ERK52R/pMAM, and ERK71R/pMAM plasmids (10 μg of DNA per electroporation) were introduced into 32D-EAK cells via electroporation by using a Gene Pulser (Bio-Rad). Population clones were selected in the presence of G418 and mycophenolic acid (25 μg/ml) for 10 to 14 days as described previously (35).

Proliferation and survivability assays. To assay the ability of EAK and EAK mutant derivatives to abrogate IL-3 dependence for proliferation in the presence of EGF, 4×10^5 log-phase wild-type and mutant EAK-expressing cells were washed in phosphate-buffered saline and seeded in 32D medium containing EGF (100 ng/ml) but lacking WEHI-3B cell-conditioned medium (source of IL-3) (29). Every 3 days, fresh medium was added to the cells. Live, trypan blue-excluding cells were counted every day to determine the number of viable cells. The same procedure was used to determine the GAS6-induced IL-3 abrogation for 32D-Axl cell proliferation. GAS6 was added to cells at 100 ng/ml. The survivability assays were carried out as described above, except that on day 9 cells were spun down and reseeded in complete 32D medium containing IL-3. Live, trypan blue-excluding cells were counted every day to determine the number of rescued, viable cells.

To assay the effects of dominant negative ERK mutants on EAK-induced IL-3-independent proliferation, EAK cells harboring pMAM, ERK52R/pMAM, and ERK71R/pMAM were seeded in IL-3-deficient 32D medium containing either 1 μM dexamethasone or dimethyl sulfoxide (dissolving agent; Sigma). Cells were then allowed to proliferate in the presence or absence of EGF and counted as described above.

Immunoprecipitation, in vitro binding assays, and Western blotting (immunoblotting). Cells were lysed on ice in lysis buffer containing 1% Triton X-100 as previously described (29). For immunoprecipitation, cell lysates were precleared with 5 μg of normal rabbit immunoglobulin G per ml and then protein A-G–agarose beads (Santa Cruz) for 1 h at 4°C. Precleared lysates were then incubated with anti-rEGFR extracellular domain antiserum (antibody 1382; 1:500; gift of H. Earp), anti-Axl immunoglobulin-like antibody (1:1,000) (32), or a monoclonal anti-Shc antibody (2 μg/ml; Transduction Laboratories) from 1 to 2 h to overnight at 4°C. Immune complexes were collected with the addition of protein A-G–agarose. Immunoprecipitates were washed three times with lysis buffer. For in vitro binding assays, 1 mg of total lysates was mixed with 10 μg of purified glutathione *S*-transferase (GST)-Grb2 SH2 fusion protein conjugated to agarose beads and incubated at 4°C for 4 h. Protein complexes were collected by centrifugation and washed three times with lysis buffer. After heat denaturation and centrifugation, eluted proteins were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto Immobilon membranes (Millipore). Western blotting with the primary antibodies indicated in Results was performed (29). Detection of immunoreactivity was accomplished with the ECL system (Amersham). For ERK mobility shift assays, 50 μg of total protein was loaded onto a 15% low-cross-linking SDS-polyacrylamide gel as described previously (6).

PI-3 kinase activity assays. For measurement of receptor-associated PI-3 kinase activity, cells were serum starved and treated with ligand as described above. Assays were performed essentially as described by Whitman et al. (54). For 32D and 32D-EAK cells, precleared lysates from 2×10^7 cells were incubated for 1 to 2 h with antiserum 1382 at 4°C. As a positive control, 32D-αPDGFR cell lysates were incubated with the anti-PDGFR α subunit antibody (Genzyme) for 1 to 2 h at 4°C (14). Protein A-G–agarose beads were then added to immune complexes for 1 h. Following three washes in ice-cold phosphate-buffered saline–1% Nonidet P-40–100 mM Na_3VO_4, three washes in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-′-2-ethanesulfonic acid; pH 7.4)–0.5 M LiCl–100 mM Na_3VO_4, and three washes in 10 mM HEPES (pH 7.4)–100 mM NaCl–1 mM EDTA–100 mM Na_3VO_4, immunoprecipitates were resuspended in 80 μl of reaction buffer (10 mM HEPES [pH 7.4], 20 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, 40 μM ATP) containing 0.25 mg of phosphatidylinositol (Avanti) per ml and [γ-^{32}P]ATP (30 μCi). The kinase reaction mixture was incubated for 20 min at 30°C with occasional agitation, and then the reaction was stopped by addition of 20 μl of 8 N HCl. Following chloroform-methanol (1:1) extractions, the chloroform phase was subjected to thin-layer chromatography analysis.

Raf-1, MEK-1, and ERK kinase assays. In vitro Raf-1, MEK-1, and ERK kinase activities were determined largely by the method described by Samuels et al. (44). In brief, immune complexes were prepared by incubating 200 to 300 μg of cell lysates with anti-Raf-1 antibody (1.5 μg; Santa Cruz), anti-MEK-1 antibody (MEK1-NT; 1:1,000 dilution; UBI), and anti-ERK1 antibody (ERK1-CT; 8 μg; UBI). Immune complexes were collected and washed in lysis buffer and then reaction buffer as previously described (41). All reaction buffers were prepared and assays were carried out as described by Samuels et al. To assay Raf-1 kinase activity, immune complexes were incubated in reaction buffer with 50 ng of the kinase-deficient MEK-1 used as a substrate. A bacterial strain expressing the kinase-deficient MEK-1 was kindly provided by J. Westwick. This protein was then purified through an Ni^{2+} column as previously described (9, 53). The in vitro kinase activity of MEK-1 was determined by carrying out a kinase reaction containing MEK-1 immune complexes in the presence of 2.5 μg of a GST-[K71R]*erk*1 agarose conjugate (UBI). In vitro ERK kinase activity was assessed by incubating immune complexes in reaction buffer containing 20 μg of myelin

basic protein (UBI) as the substrate. All reactions were analyzed on SDS-polyacrylamide gels as previously described (44).

Ras-GTP–Ras-GDP assays. Ras-GTP–Ras-GTP assays were carried out largely as previously described (17). Briefly, 32D-EAK cells were serum starved and labeled in phosphate-free RPMI 1640 supplemented with ^{32}P, at 1 mCi/2 × 10^7 cells for 4 h at 37°C. Following labeling, cells were treated with EGF at 100 ng/ml and 37°C for 10 min. Cells were lysed and $p21^{ras}$ immunoprecipitations were carried out as previously described (17). GTP and GDP were separated by thin-layer chromatography and quantitated in a PhosphorImager.

RESULTS

To elucidate the downstream components involved in Axl signaling, we used coimmunoprecipitation assays to examine potential interactions between EAK and a number of known signaling molecules in vivo, including $p21^{ras}$-GAP, Raf-1, Shc, Grb2, phospholipase C-γ, and PI-3 kinase. Of these, only the p85 subunit of the PI-3 kinase was observed to be physically associated with EAK upon ligand stimulation (see below). In addition, we examined whether EAK activation would biochemically activate transcription factor NF-κB or mitogen-activated protein kinases, including ERKs (9, 51). To assess whether NF-κB is translocated into the nucleus upon EAK activation, cell lysates were fractionated following EGF stimulation and anti-NF-κB antibody was used to localize NF-κB (1). Western blot analysis revealed that following EGF stimulation, NF-κB remained in the cytoplasm, indicating the inactive state of this protein (data not shown). Thus, the NF-κB pathway is not induced by activation of the Axl kinase. Ligand-induced EAK activation, however, was found to activate the Ras/ERK pathway. In addition, in vitro binding assays were used to further examine potential receptor-substrate interactions as presented below in detail.

Grb2 and Shc are adaptor molecules involved in EAK signaling. While EAK was not observed to coimmunoprecipitate with any of the known SH2-containing linker molecules in vivo (data not shown), a potential Grb2 binding site, YVNM (also known as PI-2; see below; 49), was located in the Axl intracellular domain. To determine whether this motif plays a role in recruiting Grb2 in EAK signaling, in vitro binding assays were performed to examine potential interactions between EAK and the SH2 domain of Grb2 fused to the GST protein (GST-Grb2 SH2). As shown in Fig. 1A, EAK associated with the SH2 domain of Grb2 in a ligand-dependent manner, indicating the potential involvement of this protein in the activation of $p21^{ras}$ in EAK signaling (20; see below). To confirm the specificity of these potential receptor-substrate interactions, we sought to determine the sequence motif of Axl required for Grb2 association and whether this association requires a functional Axl kinase domain. Two EAK derivatives containing mutations in the intracellular domain were therefore constructed. Kinase-dead mutant protein EAK567R contains a Lys→Arg substitution in the ATP binding domain, while the EAKΔPI-2 mutant protein has the putative Grb2 and PI-3 kinase binding site deleted (amino acids 821 to 824) (18, 33). Both mutant proteins were shown to be expressed on the cell surface by fluorescence-activated cell sorter (FACS) analysis at levels similar to that of wild-type EAK (data not shown) (29). As demonstrated in Fig. 1A, neither mutant bound to the SH2 domain of Grb2 molecules in vitro. Thus, the YVNM motif located in the Axl intracellular domain appears to be important for binding of both Grb2 and PI-3 kinase (see below). Both mutant proteins were also tested for the ability to be autophosphorylated in response to ligand stimulation. As expected, no autophosphorylation was detected in the kinase-dead mutant protein whereas the EAKΔPI-2 mutant protein underwent ligand-dependent autophosphorylation (see Fig. 4A).

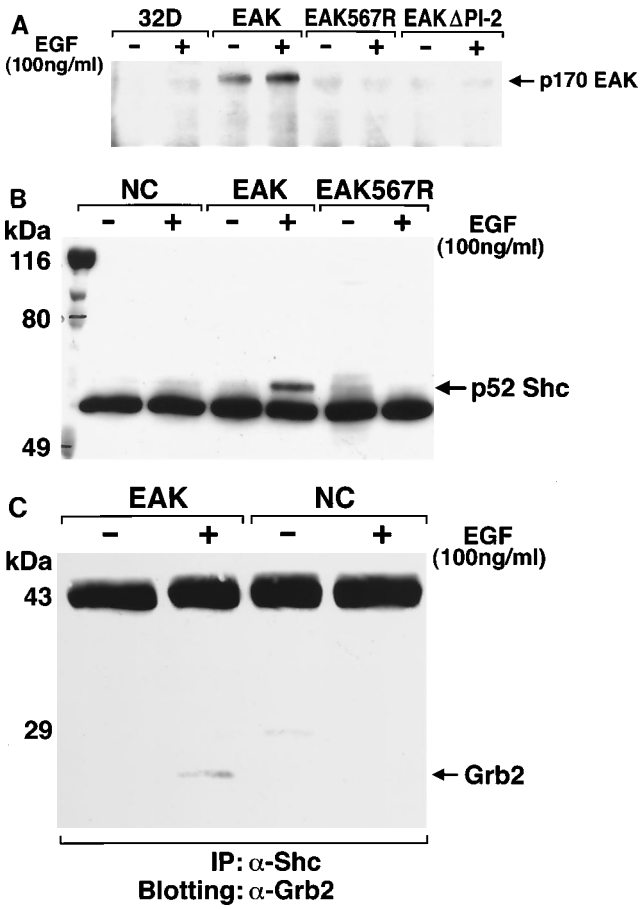

FIG. 1. Characterization of Grb2 binding and Shc-mediated signaling events following EGF stimulation in quiescent wild-type and mutant EAK-expressing 32D cells. (A) 32D cell lysates were prepared following EGF stimulation (+) or mock treatment (−) for 10 min at 37°C as described in Materials and Methods. Total cellular protein (1 mg) was mixed with purified GST-Grb2 SH2 protein, separated on an SDS–9% polyacrylamide gel, and analyzed by immunoblotting with anti-Axl antibody 17-4 (1:5,000). The position of p170 EAK is indicated. (B) Total cellular protein (800 μg) was immunoprecipitated with an anti-Shc antibody (1:125; Transduction Laboratories), separated on an SDS–12% polyacrylamide gel, and analyzed by immunoblotting with an anti-pTyr antibody (1:1,000; UBI). Molecular sizes are indicated on the left. The position of p52-phosphorylated Shc is indicated on the right. 32D cells were the parental cells. EAK567R and EAKΔPI-2 (EAK) are the kinase-dead and the Grb2 and PI-3 kinase binding site EAK mutant proteins, respectively. (C) Total cell lysates were immunoprecipitated with a monoclonal anti-Shc antibody as for panel B. Immune complexes were separated on an SDS–15% polyacrylamide gel and blotted with a monoclonal anti-Grb2 antibody at a 1:5,000 dilution (Transduction Laboratories). NC, vector-harboring 32D cells.

The adaptor protein Shc has been implicated in mediation of Ras activation downstream of several RTKs (37). As shown in Fig. 1B, p52 Shc was found to be significantly tyrosine phosphorylated in EAK-expressing cells only upon ligand stimulation. Tyrosine phosphorylation of p52 Shc is dependent upon intact EAK kinase activity, as no Shc phosphorylation was detected in the kinase-dead mutant protein-expressing cells following ligand stimulation. It has been documented that Shc proteins are tyrosine phosphorylated and associate with Grb2 upon treatment with EGF or insulin (36, 37). Since Shc does not appear to associate with EAK in a ligand-dependent manner (data not shown), we sought to determine if Shc-Grb2 association could be induced upon EAK activation and serve to recruit guanine nucleotide exchange factor mSOS for acti-

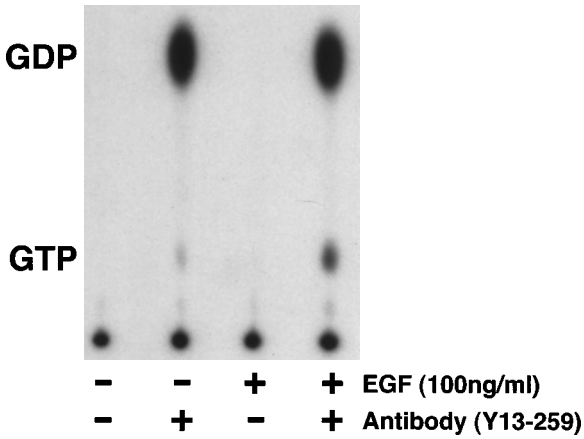

FIG. 2. Effects of EAK activation on the Ras-GTP/Ras-GDP ratio. Quiescent 32D-EAK cells were mock treated (−) or treated with EGF (+) for 10 min at 37°C. Cell lysates were prepared and incubated in the presence (+) or absence (−) of anti-Ras antibody Y13-259 (3 μg), and bound nucleotides were analyzed by thin-layer chromatography (see Materials and Methods). A representative assay is shown, and the positions of *ras*-bound GTP and *ras*-bound GDP are indicated.

vation of $p21^{ras}$ (11, 37, 40, 48). As shown in Fig. 1C, Grb2 coimmunoprecipitated with Shc in EAK-expressing cells but not in vector-harboring cells in response to foster ligand stimulation (also see Fig. 9C). Taken together, these results suggest that Grb2 can be recruited to the plasma membrane for activation of $p21^{ras}$ as an early event in EAK signaling either by direct binding to the receptor or by Shc-Grb2 complex formation triggered by Shc phosphorylation. The latter mechanism for Grb2 recruitment and Ras/ERK activation may account for the fact that lower levels of ERK activation were seen in cells expressing EAKΔPI-2, which lacks the putative Grb2 binding site (see below).

To further pursue the possibility that the recruitment of the Grb2 molecule to Shc in response to Axl kinase activation may induce $p21^{ras}$ activation, we measured in vivo Ras-GTP/Ras-GDP ratios (17). An increase in the Ras-GTP/Ras-GDP ratio of at least twofold was consistently observed in 32D-EAK cells upon foster ligand stimulation (Fig. 2). We then further characterized the response of downstream signaling molecules involved in the Ras/ERK pathway to EAK activation. To determine the activity of known serine/threonine and dual-specificity kinases downstream of $p21^{ras}$, namely, Raf-1 and MEK-1, in vitro kinase assays on enzyme-specific immune complexes were performed (44). By using kinase-deficient MEK as a substrate for Raf-1 and the GST-ERK71R fusion protein, which contains a loss-of-function mutation in the ERK1 domain, as a substrate for MEK-1, we confirmed the involvement and ligand-dependent activation of Raf-1 and MEK-1 in EAK signaling (data not shown). We then investigated whether the ERKs, downstream of MEK (24), are activated upon EAK activation. p44 and p42 ERK mobility shift assays were performed. As shown in Fig. 3, both p44 and p42 ERK bands were shifted, indicating phosphorylation and activation of these two proteins by MEK upon EAK activation. In contrast, the kinase-dead EAK mutant protein failed to activate the ERKs. As expected, lower levels of ERK activation were observed in cells expressing EAKΔPI-2. Since the putative Grb2 docking site is absent in the EAKΔPI-2 mutant protein, these results further confirmed that Shc is partially responsible for recruiting Grb2 in vivo and activating the Ras/ERK pathway following EAK activation.

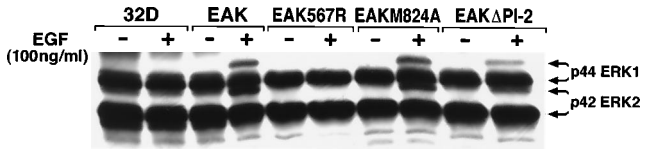

FIG. 3. Mobility shift assay of p42 and p44 ERKs in wild-type and mutant EAK-expressing 32D cells. Quiescent 32D transfectants were either mock treated (−) or treated with EGF (+) and lysed. A 50-μg sample of total protein was separated on a low-cross-linking SDS–15% polyacrylamide gel. Western blot analysis was performed with an anti-ERK1 antibody at a concentration of 0.25 μg/ml (K-23; Santa Cruz). 32D cells was the parental cells. EAK567R is the kinase-deficient EAK mutant protein. EAKM824A is the PI-3 kinase binding site mutant protein, whereas EAKΔPI-2 is deficient in both Grb2 and PI-3 kinase binding. The positions of the phosphorylated forms of ERKs p44 and p42 are indicated on the right.

Increased PI-3 kinase activity and its p85 regulatory subunit are associated with activated Axl kinase. Two putative PI-3 kinase binding sites (YALM [PI-1] and YVNM [PI-2]) have been identified in the Axl intracellular domain (18, 33, 49). To determine whether these are indeed binding sites for the p85 regulatory subunit and whether this association is important for Axl biology, potential physical interactions between EAK and p85 were examined by coimmunoprecipitation assays. As shown in Fig. 4B, p85 is associated with EAK in a ligand-dependent manner. This association is dependent upon Axl kinase activity, as no binding was detected with kinase-dead mutant protein EAK567R. Despite the physical interaction between EAK and p85, Western analysis using a phosphotyrosine antibody showed that the p85 subunit associated with the Axl kinase is not phosphorylated (data not shown). To further define the functional importance of the two putative PI-3 kinase binding sites, three deletion mutant proteins lacking either one or both binding sites, namely, EAKΔPI-1 (YALM), EAKΔPI-2 (YVNM), and EAKΔPI (YALM and YVNM), were generated. While cells harboring EAKΔPI-2 were successfully generated with comparable levels of EAK expression (Fig. 4C), we were unable to generate clones with

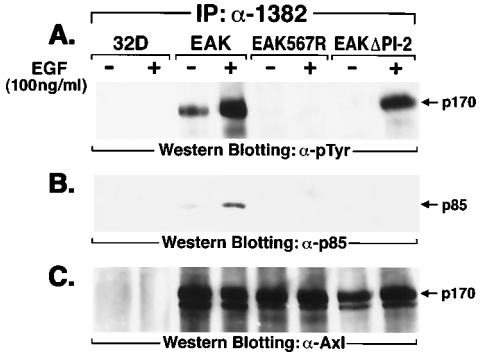

FIG. 4. Receptor autophosphorylation and p85 association of wild-type and mutant EAKs. Quiescent wild-type and mutant EAK-expressing 32D cells were either mock treated (−) or treated with EGF (+) for 10 min at 37°C. Cells were lysed, and EAK complexes were immunoprecipitated with the anti-rEGFR antibody 1382 (1:500). Western analysis was performed as described in Materials and Methods. The mutant EAK-expressing 32D cells used are described in the legend to Fig. 3. (A) The upper half of the immunoblot (cut at the 110-kDa mark) was incubated with an anti-pTyr antibody (1:1,000; UBI). The 170-kDa EAK chimeric receptor is indicated. (B) The lower half of the immunoblot was incubated with a monoclonal anti-p85 antibody (1:1,000; UBI), showing that p85 binding is dependent upon an active EAK. The position of the p85 subunit of the PI-3 kinase is indicated. (C) To quantitate the amount of total protein loaded in each lane, the pTyr immunoblot was stripped and reprobed with anti-Axl C-terminal antibody 17-4 (1:5,000).

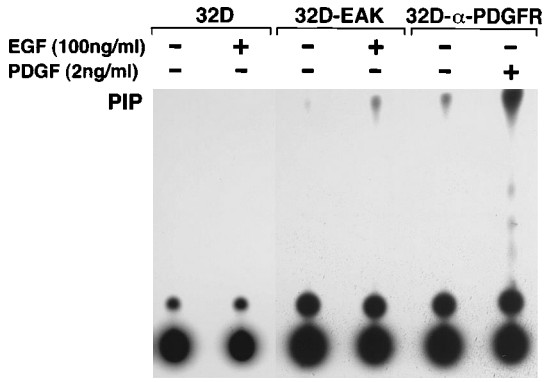

FIG. 5. Coimmunoprecipitation of PI-3 kinase activity with EAK in 32D cells. Quiescent 32D, 32D-EAK, and 32D-αPDGFR cells (positive control) were either mock treated (−) or treated with the indicated growth factor (+) for 10 min at 37°C. Cell lysates were prepared, and 1 mg of total cellular protein was immunoprecipitated with either antibody 1382 (32D and 32D-EAK cells) or the anti-human αPDGFR antibody (32D-αPDGFR cells) as described in Materials and Methods. Immune complexes were then subjected to a PI-3 kinase assay (see Materials and Methods). The position of PI-3 phosphate (PIP) is indicated. A representative result of three independent experiments is shown.

sufficiently high levels of expression of the EAKΔPI-1 and EAKΔPI proteins for direct comparison with wild-type EAK in binding assays. Since the PI-2 site represents a better consensus for PI-3 kinase binding (49), we focused only on EAKΔPI-2-expressing cells for the subsequent biochemical and biological studies. As shown in Fig. 4A and B, the EAKΔPI-2 mutant protein failed to bind to p85, although it still undergoes ligand-dependent autophosphorylation. To determine the functional consequences of p85 binding to EAK, we measured the PI-3 kinase activity associated with the Axl kinase in response to foster ligand stimulation (54). As shown in Fig. 5, an average of a two- to threefold increase in PI-3 kinase activity associated with EAK was observed after ligand stimulation, indicating that the recruitment of PI-3 kinase by the Axl kinase activates this lipid kinase.

As mentioned above, the putative PI-2 site, YVNM, may also be a binding site for Grb2 molecules (49). Thus, biochemical and biological consequences observed as a result of EAKΔPI-2 mutant signaling may be influenced by both the Ras/ERK and PI-3 kinase pathways. To dissect the involvement of PI-3 kinase in EAK signaling, we constructed EAK mutant protein EAKM824A, which contains a Met→Ala substitution at the +3 position in the YVNM motif (YVNA). Since the methionine residue at the +3 position is known to be critical for PI-3 kinase binding, this mutant protein should affect PI-3 kinase binding but not Grb2 docking (19, 49). By FACS analysis, we observed the expression of this mutant protein on the cell surface at about 70% of that of EAK. As will be discussed below, the reduced level of EAKM824A expression does not appear to impair the ability of receptor-mediated, IL-3-independent proliferation in 32D cells. As shown in Fig. 6B, mutant protein EAKM824A failed to bind to the p85 subunit of PI-3 kinase yet underwent ligand-dependent autophosphorylation (Fig. 6A). Consistent with the FACS analysis, slightly lower levels of EAKM824A than wild-type receptor expression were observed (Fig. 6C). Because lower levels of EAKM824A are expressed in these cells, we exposed these gels for an extended period of time and found no association between p85 and EAKM824A (data not shown). Furthermore, the Ras/ERK pathway remains intact in EAKM824A-expressing cells, as demonstrated by the ability of the EAKM824A mutant protein to bind GST-Grb2 SH2 in vitro, to activate p52 Shc, to promote Shc-Grb2 complex formation (data not shown), and to induce ERK activation Fig. 3).

EAK-induced proliferation is associated with full activation of the Ras/ERK pathway. We have previously demonstrated that EAK, when activated by EGF in IL-3-dependent 32D cells, can abrogate the IL-3 dependence for proliferation (29). To further characterize the role of individual pathways in Axl biology, 32D-EAK mutants deficient in either intrinsic kinase activity or p85 binding were subjected to EGF-dependent growth assays in the absence of IL-3. These cells were stimulated with EGF to determine whether these mutants would respond to the foster ligand for proliferation in the absence of IL-3. As shown in Fig. 7A, 32D cells expressing the EAKM824A mutant protein lacking the binding site for the PI-3 kinase p85 subunit were capable of undergoing IL-3-independent proliferation as efficiently as wild-type EAK-expressing cells. By contrast, EAK567R- and EAKΔPI-2-expressing cells failed to respond to EGF stimulation for growth in the absence of IL-3. Since Ras/ERK activation is partially impaired in EAKΔPI-2-expressing cells, these results further demonstrate that both the kinase activity and an intact Ras/ERK pathway are essential for EAK-induced mitogenesis in 32D cells. However, the fact that cells expressing the EAKM824A mutant protein are fully capable of proliferating in the absence of IL-3 suggests that the PI-3 kinase pathway is dispensable in EAK-induced mitogenesis.

Although certain oncogenes do not induce proliferation in 32D cells, they exert their action by blocking programmed cell death that results upon cytokine withdrawal. To determine whether any of the mutants which failed to elicit IL-3-independent proliferation could sustain survival in the absence of IL-3, cells were rescued by using IL-3 after 9 days of IL-3 starvation. As shown in Fig. 7B, EAKΔPI-2-expressing cells were capable of survival for 9 days of IL-3 deprivation only when stimulated with EGF, whereas kinase-dead EAK567R mutant protein-expressing cells failed to be rescued by IL-3. These results further indicate that full activation of the Ras/ERK signaling

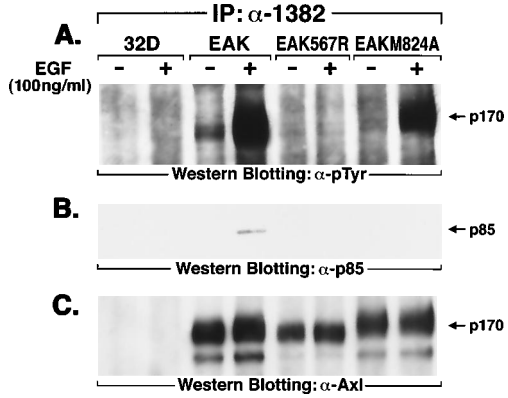

FIG. 6. Characterization of receptor autophosphorylation and p85 association of the EAKM824A mutant protein. Quiescent wild-type and mutant EAK-expressing 32D cells were either mock treated (−) or treated with EGF (+) for 10 min at 37°C. Cells were lysed, and EAK complexes were immunoprecipitated with anti-rEGFR antibody 1382 (1:500). Western analysis was performed as described in Materials and Methods. The mutant EAK-expressing 32D cells used are described in the legend to Fig. 3. (A) The upper half of the immunoblot (cut at the 110-kDa mark) was incubated with an anti-pTyr antibody (1:1,000; UBI). The position of the 170-kDa EAK chimeric receptor (p170) is indicated. (B) The lower half of the immunoblot was incubated with a monoclonal anti-p85 antibody (1:1,000; UBI), showing that p85 binding is dependent upon an active EAK. The position of the p85 subunit of the PI-3 kinase is indicated. (C) To quantitate the amount of total protein loaded in each lane, the pTyr immunoblot was stripped and reprobed with anti-Axl C-terminal antibody 17-4 (1:5,000).

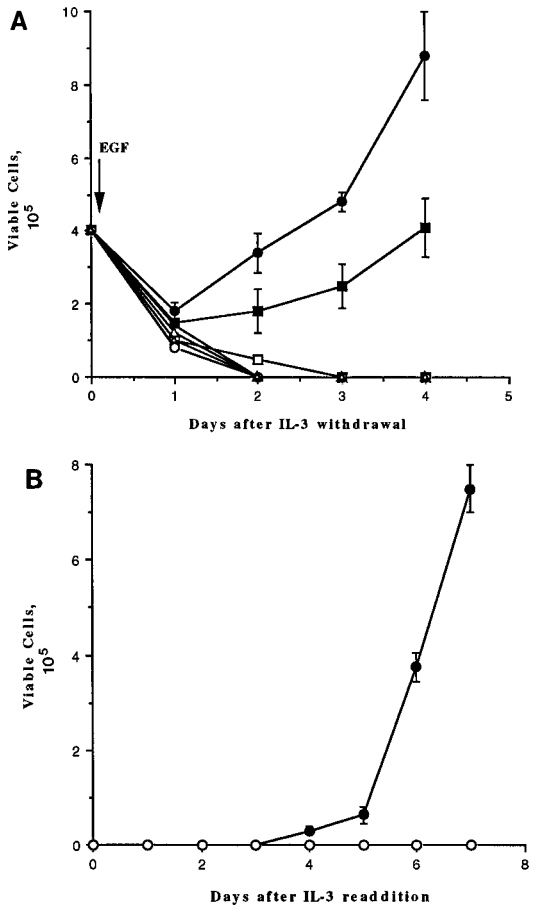

FIG. 7. Proliferation and rescue assays of 32D cells expressing wild-type and mutant EAKs in response to stimulation with EGF. Cells (4×10^5) were seeded in RPMI 1640 medium containing 15% fetal bovine serum in the presence or absence of EGF (100 ng/ml). Fresh medium supplemented with EGF was added every third day. (A) Proliferation assay. Live, proliferative cells were counted every day on the basis of the trypan blue exclusion method. These results show that EAK-induced proliferation is dependent upon EAK kinase activity and full activation of the Ras/ERK pathway but not PI-3 kinase subunit p85 binding. Symbols: ■, EAK plus EGF; ●, EAKM824A plus EGF; ▲, EAKΔPI-2 plus EGF; ▲, EAK567R plus EGF; □, EAK minus EGF; ○, EAKM824A minus EGF; △, EAKΔPI-2 minus EGF; △, EAK567R minus EGF. (B) Following 9 days of EGF treatment, wild-type and mutant EAK-expressing cells were spun down and reseeded in complete 32D medium containing IL-3. Since cells expressing wild-type EAK and EAKM824A proliferate in IL-3-deficient medium supplemented with EGF, only results for other EAK mutants are shown. These data demonstrate that EAKΔPI-2 is capable of sustaining 32D cell survival after IL-3 withdrawal. Results are shown as the means and standard deviations of three replicates per point. The mutant proteins tested were EAKΔPI-2 (Grb2 and p85 binding deficient), EAK567R (kinase dead), and EAKM824A (p85 binding deficient). Symbols: ●, EAK plus EGF; ○, Axl plus GAS6.

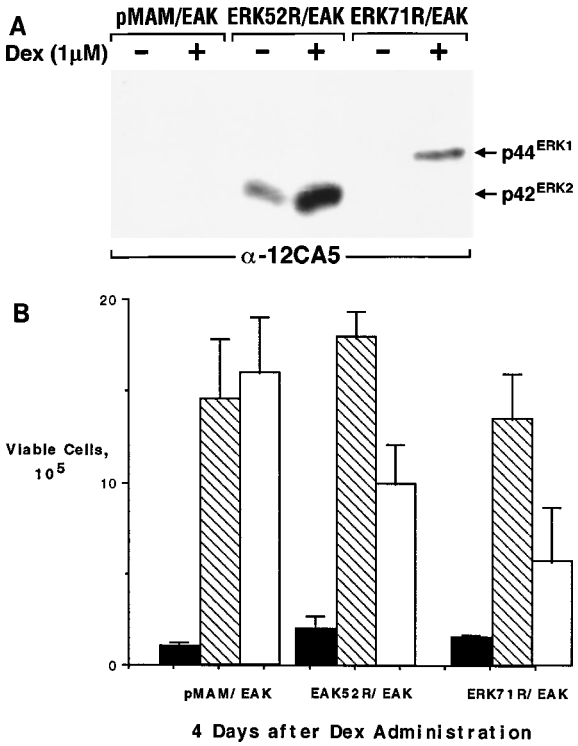

FIG. 8. Effects of dominant negative ERK mutants on EAK-induced proliferation of 32D cells in the absence of IL-3. (A) Dexamethasone (Dex)-induced expression of the ERK71R and ERK52R constructions. 32D-EAK cells expressing either the parental pMAM vector or the HA-tagged ERK71R and ERK52R constructions were treated with dimethyl sulfoxide (−) or 1 μM dexamethasone (+) for 24 h at 37°C. Cells were then lysed and subjected to Western analysis, as described in Materials and Methods, with an anti-HA antibody (12CA5; Boehringer Mannheim) at a concentration of 2 μg/ml. The positions of the p44 ERK1 and p42 ERK2 mutant proteins are indicated. (B) Assay of proliferation of 32D-EAK cells expressing the dominant negative ERKs. Cells (4×10^5) were seeded in RPMI 1640 medium containing 15% fetal bovine serum supplemented with EGF (100 ng/ml) in the presence or absence of dexamethasone (1 μM). Fresh medium supplemented with EGF and dexamethasone was added every third day. Live, trypan blue-excluding, proliferative cells were counted every day. Results are shown as the means and standard deviations of three replicates per point. ■, dexamethasone; ▨, EGF; □, dexamethasone plus EGF.

pathway is essential for EAK-induced, IL-3-independent proliferation in 32D cells. However, a lower threshold of Ras/ERK activation is sufficient for the survival of these cells.

To confirm the importance of the Ras/ERK pathway in EAK-induced proliferation, molecules interfering with the function of the $p21^{ras}$/ERK pathway were introduced into 32D-EAK cells. First, HA-tagged, dominant negative ERK52R (ERK2) and ERK71R (ERK1) mutant proteins were expressed under the control of the mouse mammary tumor virus long terminal repeat-inducible promoter in 32D-EAK cells (41). As shown in Fig. 8A, dexamethasone-induced ERK52R and ERK71R expression was observed. Following dexamethasone induction, blockage of EGF-induced activation of the endogenous ERKs in 32D-EAK cells expressing the ERK52R and ERK71R constructions was observed (data not shown). Upon dexamethasone induction, EAK cells expressing either the ERK52R or the ERK71R construction failed to respond fully to EGF-induced proliferation, whereas EGF-induced proliferation in control EAK cells was not affected (Fig. 8B). The functional redundancy of the p44 and p42 ERKs and the fact that other pathways may be involved in EAK-mediated growth activities may explain why only 40 to 50% inhibition of EGF-dependent proliferation was observed in these cells (9). A farnesyltransferase inhibitor which blocks $p21^{ras}$-dependent signal transduction transformation was administered to 32D-EAK cells to determine whether this inhibitor affects EAK-induced proliferation (6, 22). Inhibition of EAK-mediated proliferation to the level induced by the dominant negative ERK mutants was achieved by this Ras inhibitor (data not shown). These results further demonstrate that the Ras/ERK pathway is involved in the mitogenic activity of EAK in 32D cells.

GAS6-Axl interactions result in downstream signaling events distinct from EAK-mediated signal transduction. We have initiated studies of the chimeric receptor EAK in the hope of understanding the mechanism whereby the activated

Axl kinase transduces signals. Concomitant with this work, an intensive effort has been made to identify the cognate ligand for Axl. Very recently, we have identified GAS6 as an Axl ligand (52). GAS6 is homologous to vitamin K-dependent anticoagulation factor protein S, and its expression is dramatically increased in quiescent NIH 3T3 cells (25). The biological function of GAS6 is unknown, although this ligand is weakly mitogenic in only a few Axl-expressing cell lines (8a). As purified GAS6 became available, we initiated biochemical analyses similar to the work presented above for EAK studies to determine downstream events resulting from GAS6-Axl interactions. Although GAS6 stimulation resulted in Axl autophosphorylation in 32D cells (Fig. 9A) with an intensity comparable to that caused by EGF stimulation in EAK-expressing cells, this Axl activation did not result in activation of the Ras/ERK pathway, as was seen in EAK-expressing cells after EGF stimulation (Fig. 9B and C). This conclusion is based on two findings: the absence of Shc phosphorylation and Shc-Grb2 complex formation and the lack of ERK mobility shift after GAS6 administration. On the other hand, the p85 subunit of the PI-3 kinase is associated with the autophosphorylated Axl kinase in a GAS6-dependent fashion, similar to the findings in the EAK system (Fig. 9D). To rule out the trivial possibility that the differences in downstream signaling observed between EAK and Axl were due to lower levels of Axl expression, levels of receptor expression were determined and found to be comparable (Fig. 9E).

Given the differences in activation of the biochemical pathways, we examined the ability of the GAS6-Axl interaction to induce a mitogenic response. To this end, we assessed the ability of Axl-transfected 32D cells to abrogate dependence on IL-3 for proliferation in response to GAS6. 32D-Axl cells were seeded in medium containing GAS6 but not IL-3. In contrast to the effect of EGF on 32D-EAK cells, no proliferation was observed in 32D-Axl cells treated with GAS6 (Fig. 10). In dose-response experiments, no proliferation of Axl-32D cells was observed, even in the presence of 500 ng of GAS6 per ml (data not shown). Despite the absence of a mitogenic response, it was possible that GAS6 activation of Axl would sustain survival of 32D-Axl cells upon IL-3 withdrawal. When this was tested in a stringent 9-day IL-3 withdrawal assay, no 32D-Axl cells survived (data not shown). Taken together, these results suggest that GAS6 stimulation of the full-length Axl receptor kinase results in cellular responses distinct from those of the chimeric EGFR-Axl receptor kinase.

DISCUSSION

Biological and biochemical characterization of chimeric receptors consisting of a known extracellular ligand binding domain and a kinase domain from an orphan receptor has been proven to be a fruitful approach for studying the function of tyrosine kinases (45, 55). The assumption that chimeric receptors possess the same biochemical and biological properties as the wild-type counterparts from which the intracellular domain was derived has been validated in a number of studies (45, 55). For example, an EGFR-nerve growth factor receptor chimera was able to induce neurite outgrowth in response to EGF in rat pheochromocytoma PC12 cells. Bioassays have demonstrated a good concordance between the behavior of the EGFR-*ret* chimera and that of the *RET*/*PTC* oncogene, an activated version of *ret*. In addition, similar patterns of substrate tyrosine phosphorylation by EGFR/erbB-2 and by constitutively active $gp185^{erbB\text{-}2}$ have been reported. To initially dissect the functional requirements responsible for Axl-induced proliferation of the IL-3-dependent myeloid cell line 32D, we have used an EGFR-Axl chimera (EAK) to identify downstream signaling components resulting from the foster ligand-receptor interaction (29). Our results demonstrated that differential activation of the Ras/ERK pathway results in distinct biological consequences. As shown by growth and survivability assays of a panel of EAK derivatives, EAK-induced mitogenesis requires full activation of the Ras/ERK pathway whereas partial activation of this pathway is sufficient for cell survival. Although EAK-associated PI-3 kinase activity appeared to increase moderately upon EGF stimulation, the biological function of this lipid kinase in EAK-induced signaling remains unclear.

As an early event in $p21^{ras}$ activation, we have shown that two functional routes are responsible for recruiting Grb2 adaptor molecules for $p21^{ras}$ activation. First, we have shown ligand-dependent EAK-Grb2 association by in vitro binding assays. Secondly, although EAK does not appear to directly associate with p52 Shc molecules, EAK activation nevertheless stimulates tyrosine phosphorylation of p52 Shc and complex formation between Shc and Grb2, a hallmark for recruitment of mSOS for $p21^{ras}$ activation on the plasma membrane (20, 37). Since no ligand-dependent physical interaction between EAK and p52 Shc was observed by coimmunoprecipitation and in vitro binding assays, an intriguing possibility is that p52 Shc is phosphorylated by an unidentified substrate of EAK. Consistent with our observation that Shc phosphorylation and Shc-Grb2 complex formation are early events in the activation of the Ras/ERK pathway is the recent report that the phosphorylation of Shc and the binding of Grb2 to Shc are the prerequisites for IL-4-induced $p21^{ras}$ activation (38). In addition, as seen in other cases of receptor-mediated Ras/ERK activation, EAK activation induces $p21^{ras}$-dependent events, including activation of Raf-1, MEK-1, and ERKs (Fig. 3) (28, 30).

Two potential p85 binding sites identified in the Axl intracellular domain prompted us to investigate the involvement of PI-3 kinase in Axl biology. We have shown that the p85 subunit is recruited to the Axl kinase domain in a ligand-dependent fashion (Fig. 4). By deletion mutagenesis, we identified one binding site as YVNM (PI-2; amino acids 821 to 824), located near the C terminus of the Axl protein. Another potential p85 binding site, YALM (PI-1), is located N terminal of the PI-2 binding site in the Axl intracellular domain. Although several attempts were made to determine whether this site is also important for p85 binding, transfectants bearing either EAKΔPI-1 alone or EAKΔPI (both sites deleted) expressed the mutant proteins at very low levels, perhaps because of decreased stability of the recombinant proteins. Since this poor expression essentially rendered subsequent biochemical analyses impossible, the importance of the potential N-terminal p85 binding site is unknown.

As is the case for several other RTKs, the PI-3 kinase associated with the Axl kinase appears not to be tyrosine phosphorylated (4, 51). While it has been documented that tyrosine phosphorylation of p85 may not change its catalytic activity, binding of p85 to the tyrosine-phosphorylated peptides present in the PDGFR and insulin receptor substrate 1 (IRS-1) leads to a small increase in PI-3 kinase activity (42). Consistent with these findings, we observed a two- to threefold increase in PI-3 kinase activity following foster ligand activation of EAK (Fig. 5).

The PI-3 kinase pathway has been implicated in growth factor-induced proliferation, membrane ruffling, and differentiation (reference 19 and references therein). At least two downstream targets of the PI-3 kinase have been identified (3, 8). Association of PI-3 kinase activity with the activated PDGFR and the activated colony-stimulating factor 1 receptor is correlated with their mitogenic potential (46, 47). The p110

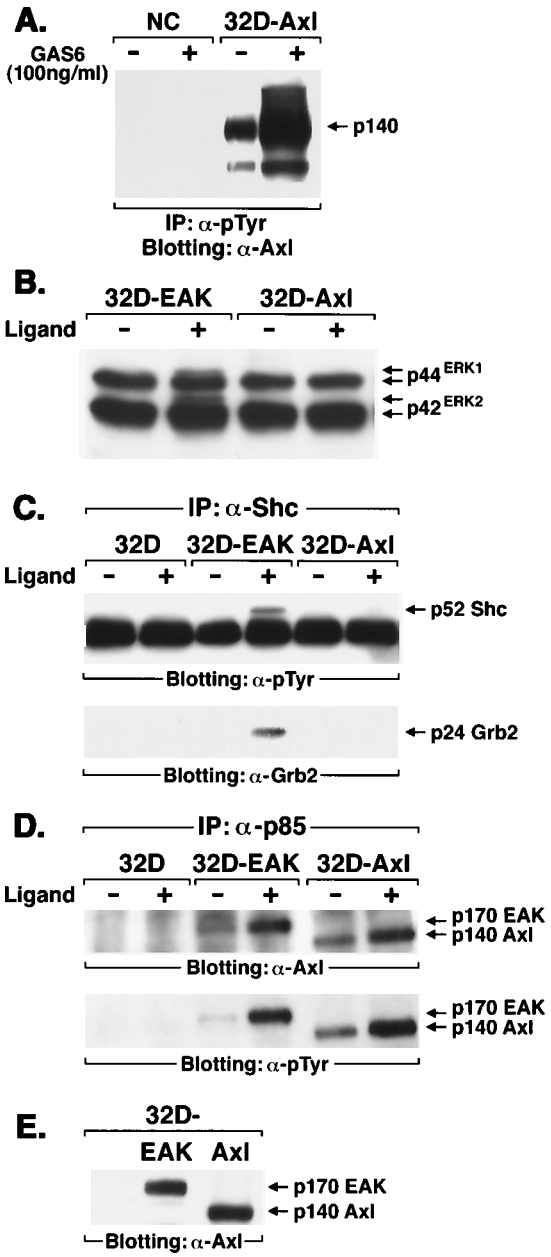

FIG. 9. Biochemical characterization of downstream events following GAS6-Axl interactions. (A) Quiescent 32D-Axl or vector-harboring 32D cells (NC) were stimulated with GAS6 (100 ng/ml) for 10 min at 37°C. Cells were lysed and subjected to immunoprecipitation with an anti-pTyr antibody (1:1,000; UBI) as described in Materials and Methods. Subsequent Western analysis was performed with anti-Axl antibody 17-4 (1:5,000). The position of p140 Axl is indicated. (B) Following either EGF (EAK) or GAS6 (Axl) stimulation, 50 μg of total cellular protein was subjected to Western analysis with anti-ERK1 antibody K-23 (Santa Cruz) at a concentration of 0.25 μg/ml. Positions of unshifted and shifted p44 and p42 ERKs are indicated. (C) Following ligand treatment (either EGF for 32D-EAK cells or GAS6 for 32D and 32D-Axl cells), cells were lysed and subjected to immunoprecipitation with an anti-Shc antibody as described in Materials and Methods. Following immunoblotting and transfer, the blotted membrane was cut at the 39-kDa mark. The top half of the membrane was incubated with an anti-pTyr antibody (1:1,000; UBI), and the bottom half of the membrane was incubated with an anti-Grb2 antibody (1:5,000; Transduction Laboratories). The positions of phosphorylated p52 Shc and Shc-associated Grb2 are indicated. (D) Following ligand treatment (either EGF for 32D-EAK cells or GAS6 for 32D and 32D-Axl cells), cells were lysed and subjected to immunoprecipitation with an anti-p85 antibody (1:1,000; UBI). Following immunoblotting and transfer, the blotted membrane was incubated with anti-Axl antibody 17-4 (1:5,000). This membrane was then stripped and reprobed with an anti-pTyr antibody (1:1,000; UBI). These data suggest that p85 coimmunoprecipitates with the activated kinase domain of either Axl or EAK. The positions of p170 EAK and p140 Axl are indicated. (E) Total cellular proteins (50 μg) of parental and EAK- and Axl-expressing 32D cells were prepared (see Materials and Methods) and separated on an SDS–9% polyacrylamide gel. After protein transfer, the membrane was blotted with anti-Axl antibody 17-4 (1:5,000). Comparable levels of receptor expression were detected.

catalytic subunit of this lipid kinase is homologous to the yeast Vps34 protein and has been suggested to play a role in vesicular trafficking (16, 19). Furthermore, this p110 subunit has recently been shown to be coupled with the effector binding domain of the activated form of $p21^{ras}$ (16, 42). The finding that *ras* (Asn-17) expression blocked the growth factor-induced accumulation of 3′ phosphorylated phosphoinositides provides a potential model for regulation of the PI-3 kinase by the Ras pathway. Our data, however, revealed no measurable mitogenic potential of the PI-3 kinase associated with the activated EAK. Cells expressing PI-3 kinase binding site mutant protein EAKM824A proliferated in response to EGF in a manner indistinguishable from that of wild-type EAK-expressing cells (Fig. 7A). Furthermore, PI-3 kinase activity assays revealed that in EAKM824A-expressing cells the overall activity of the PI-3 kinase remains unchanged upon stimulation with a foster ligand (data not shown). These results rule out the possibility that PI-3 kinase indirectly plays a role downstream of EAK signaling.

Since EAK can signal through the Ras/ERK pathway in 32D cells, we assessed the potential role of this pathway for the biological response in EAK signaling. Previously we have determined that EGF-induced activation of EAK is mitogenic in 32D cells (29). By using a panel of mutant proteins and biochemical inhibitors, we found that full activation of the Ras/ERK process is necessary for proliferation. As mentioned above, EAK mutant proteins defective in p85 binding (EAKM 824A) are able to fully activate Ras/ERK and induce proliferation. That ERK activation is important for EAK-induced mitogenesis is seen in the ability of the dominant negative ERK52R or ERK71R construction to block EAK-mediated proliferation by 40 to 50%. This notion is further strengthened by the fact that blockage of Ras function by the farnesyltransferase inhibitor impairs EAK-induced proliferation (data not shown).

32D is a myeloid cell line that proliferates in response to cytokine signaling and undergoes programmed cell death upon cytokine withdrawal. Because of these characteristics, 32D cells have been used not only to assess the mitogenic properties but also to investigate their capacity to block apoptosis. It was in similar cells that the antiapoptotic action of bcl-2 was uncovered (21). By using our Axl mutants, we assessed the role of the Ras/ERK pathway in sustaining cell survival upon IL-3 withdrawal and found that partial activation of this pathway is sufficient for EAK-induced cell survival. This is evidenced by the ability of EAKΔPI-2, which only partially activates the Ras/ERK cascade, to sustain 32D cell growth upon IL-3 withdrawal (Fig. 7B). Therefore, EAK is capable of blocking apoptosis via partial activation of the Ras/ERK pathway. Through these studies, we have determined that two levels of the Ras/ERK action are operative in EAK signaling. Full Ras/ERK activation is necessary for EAK-induced proliferation, whereas a lower threshold of this activation is required to block apoptosis following EAK activation.

Recently, we and others have identified a ligand for Axl as GAS6 (12, 50, 52). With the ligand at hand, we were interested in the biology and the downstream signaling pathways resulting from the bona fide Axl ligand-receptor interaction. We found

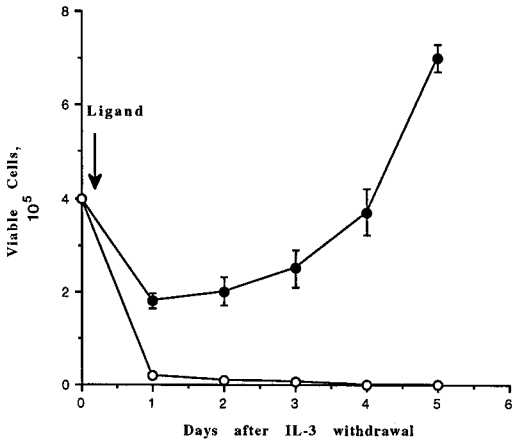

FIG. 10. Assay of 32D-EAK and 32D-Axl cell proliferation. Cells (4×10^5) were seeded in RPMI 1640 medium containing 15% fetal bovine serum in the presence of EGF (100 ng/ml) or GAS6 (100 ng/ml). Fresh medium supplemented with the indicated ligand was added every third day. Live, proliferative cells were counted every day by the trypan blue exclusion method. These data demonstrate that 32D-Axl cells are not capable of proliferating in response to GAS6 after IL-3 withdrawal. Results are shown as the means and standard deviations of three replicates per point. Symbols: ●, 32D-EAK; ○, 32D-Axl.

that in Axl-transfected 32D cells, GAS6 stimulation induces ligand-dependent Axl autophosphorylation (Fig. 9A). Previously, we have shown that GAS6 can bind to the receptor and induce autophosphorylation and internalization of the Axl receptor (52). However, ligand application to 32D-Axl cells did not induce proliferation, even at a high ligand concentration (Fig. 10; data not shown). This is in accordance with our earlier finding that GAS6 is a weak mitogen for some cells but has no growth-promoting effect on others (52). For a biochemical explanation for this observation, we assessed the Ras/ERK cascade and PI-3 kinase binding and found that although Axl kinase bound to p85 upon GAS6 stimulation, the Ras/ERK process was not activated, as evaluated by both ERK mobility shift and p52 Shc phosphorylation assays and Shc-Grb2 complex formation (Fig. 9B, C, and D). These results are consistent with our findings mentioned above that the Ras/ERK cascade is necessary for ligand-dependent mitogenesis but that the PI-3 kinase pathway is dispensable. Taken together, these data suggest that the GAS6-Axl interaction may not induce proliferation, and its primary role remains unclear. In addition, the nature of the extracellular domain can influence the intracellular signaling pathways used by the Axl kinase.

We propose two potential models invoking unconventional ligand-receptor interactions that may account for the disparate biological outcomes of signaling between Axl and EAK. The first model involves receptor heterodimerization upon GAS6 stimulation. Because the extracellular domain of the EGFR is known to induce homodimerization upon EGF stimulation when expressed in 32D cells (36a), EAK-EAK homodimers are the result of foster ligand stimulation. This homodimerization is also likely to be accomplished by surface coupling when Axl is dramatically overexpressed, as was demonstrated in our Axl-transformed 3T3 cells (33). Supporting this possibility is the finding that very high levels of Axl expression are able to transform 3T3 cells, an effect which is associated with constitutive activation of ERKs (8a). In either case, the Axl kinase is thought to support mitogenesis through activation of the Ras/ERK pathway. In GAS6-Axl interactions, however, GAS6 may induce heterodimerization of Axl with another receptor that has yet to be identified. It has been shown that heterodimerization occurs between homologs in the EGFR family (5; references therein). For example, upon binding of the ligand heregulin (or Neu differentiation factor), heterodimers form between ErbB2 and ErbB3 and between ErbB2 and ErbB4. It is thought that heterodimerization between homologous receptors may determine what SH2-containing molecules will be recruited for downstream signaling (5, 7). In GAS6-Axl signaling, potentially following a heterodimerization event, a different subset of phosphorylated tyrosines may be generated; this differs from the tyrosine phosphorylation induced by EGF-EAK interactions. In the PDGFR subfamily, PDGFRα/β heterodimerization can be induced by either PDGF-AB or PDGF-BB stimulation (15, 43). The mitogenic signal elicited following activation of PDGFR heterodimers is stronger than that induced by PDGFR homodimers (43). The fact that families of structurally related RTKs and ligands exist makes it possible that homodimerization and heterodimerization may occur dependent upon the level and type of related RTKs on the cell surface. This heterogeneity would function to widen the spectrum of biological responses resulting from a given family of receptors. As mentioned above, a number of Axl-related RTKs have been identified (2, 10, 13, 23, 34). Therefore, it is possible that heterodimerization between Axl homologs serves to modulate and diversify downstream signals by differentially recruiting SH2-containing molecules.

An alternate mechanism of Axl activation by GAS6 stimulation, however, may involve homophilic binding via the extracellular domain of Axl. Recent studies have demonstrated that the murine homolog of Axl, ARK, is capable of promoting cell aggregation through homophilic binding (2). This homophilic association, which is putatively characterized by head-to-tail dimerization of the extracellular domains, results in an increase in receptor phosphorylation. The only other RTK that has been shown to undergo homophilic binding is *DTrk*, a *Drosophila* RTK highly related to the *trk* family of mammalian neurotrophin receptors (39). The Ca^{2+}-independent, homophilic association of *DTrk* induces receptor phosphorylation and is thought to be the mechanism by which *DTrk* regulates neuronal recognition during development. The conformational change following homophilic interactions may activate a limited aspect of the kinase and therefore result in activation of fewer intracellular signaling partners. Our preliminary results have indicated that the extracellular domain of Axl can serve as a partner for the adhesion of Axl-expressing fibroblasts and 32D cells (unpublished data). Because homophilic interactions may not result in dimerization of the kinase domains of RTKs, the single activated Axl cytoplasmic domain may interact with a limited number of downstream signaling partners. This may also explain the differential involvement of the downstream components in signaling between EAK and Axl.

In conclusion, our studies on Axl signaling have uncovered potentially intricate ligand-receptor interactions depending upon the dimeric nature of the extracellular domain following ligand stimulation. The same activated Axl kinase in EAK results in signaling events and biological responses dramatically different from those of full-length Axl. While the biological role of GAS6-Axl interactions remains to be determined, our studies underscore the complexity of ligand-receptor interactions.

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