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GAS6 Mediates Adhesion of Cells Expressing the Receptor Tyrosine Kinase Axl*

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Axl is a receptor tyrosine kinase that contains both immunoglobulin and fibronectin III repeats in its extracellular domain reminiscent of cell adhesion molecules. Expression of the receptor tyrosine kinase Axl in the 32D myeloid cell line permits aggregation of cells in response to treatment with the native ligand GAS6; this aggregation was not observed in untreated 32D-Axl cells nor in treated parental cells. This aggregation can be blocked by the addition of excess Axl extracellular domain peptide and does not require intracellular Axl kinase activity. Cell surface binding activity of GAS6 was mapped to distinct plasma membrane interacting domains that are separate from the GAS6 motifs that engage the Axl receptor. This suggests that aggregation is mediated by a heterotypic intercellular mechanism whereby cell-bound GAS6 interacts with Axl receptor on an adjacent cell. This mechanism is supported by our observation that GAS6 binds to 32D parental cells which then permits their aggregation with untreated 32D-Axl cells. We have recently demonstrated that the GAS6-Axl interaction does not initiate mitogenesis in 32D cells. When considered with the adhesion results, these data suggest that an important biological function of the Axl-GAS6 interaction is to mediate cell-cell binding.

Cell adhesion molecules mediate the maintenance of tissue structures in adult organisms and morphogenesis in developing embryos. For example, E-cadherin is necessary for tight association in adult epithelium and is involved in the compaction of mesenchymal cells into a polarized epithelium (1). In addition to providing the physical means for cell assembly, these adhesion molecules also function, in part, to transduce signals from extracellular components such as the (2). Although structurally diverse, many cell adhesion molecules exhibit similarities to the immunoglobulins (Igs) and thus have been classified in the Ig superfamily of genes. Specifically, a general arrangement involving Ig motifs in the NH_2 terminus followed by fibronectin III domains in the COOH terminus of the extracellular region characterizes a subgroup of these adhesion molecules (1, 2). Included in this group are molecules such as the neural cell adhesion molecule, which is involved in the proper migration of neural crest cells, and protein phosphatase-κ and -μ, which possess intrinsic enzymatic function.

We have previously identified a novel transforming receptor tyrosine kinase, Axl (3), which contains a similar organization of Ig and fibronectin III motifs in its extracellular domain (ECD).[1] Since the original description of Axl, several other related genes with transforming potential (Sky and Mer) have been cloned (4, 5). All members of this Axl family of receptor tyrosine kinases harbor a similar ECD architecture, raising the possibility that Axl and its relatives may function in cell adhesion. That Axl may be a cell adhesion receptor was suggested by experiments using the murine Axl homolog, Ark. S2 insect cells overexpressing Ark formed multicellular aggregates that could be blocked by excess recombinant Ark ECD (6). Similar results could be achieved upon overexpression of D-Trk or protein phosphatase-κ and -μ in the same S2 cells (7–9). These data raise the possibility that receptor tyrosine kinases and transmembrane phosphatases may function both as adhesion molecules and as elements of the signaling cascade.

A ligand for Axl has been characterized by its ability to induce Axl autophosphorylation, directly bind the receptor, and cause receptor down-regulation (10). This polypeptide is the product of the growth arrest-specific gene 6 (GAS6) and was originally cloned from serum-starved NIH 3T3 cells (11). GAS6 is composed of several structural motifs including a Gla domain, EGF repeats, and G domains. The G domains, which have homology to the steroid hormone-binding globular protein, are sufficient to induce autophosphorylation of both the Axl and Rse receptors (12). The Gla domain of GAS6 is thought to bind to negatively charged phospholipids, whereas the EGF repeats can interact with integral membrane proteins; both interactions are dependent upon extracellular calcium (13, 14). GAS6 contains sites for β-hydroxylation of Asp and Asn which can also mediate protein-protein interactions (15). As Axl is a transforming receptor tyrosine kinase it was initially anticipated that its ligand, GAS6, would be a potent growth factor for Axl-expressing cells. However, although GAS6 acts as a weak mitogen in 3T3 cells (16) it is not mitogenic in other Axl-expressing cell lines. Moreover, exposure of 32D cells with ectopic expression of the Axl receptor to soluble recombinant GAS6 does not appear to induce intracellular proliferation signals (17). Thus, despite the fact that GAS6 acts as a ligand for Axl, the primary biological function of this interaction remains unclear.

The homotypic aggregation of Ark-expressing S2 cells led us to explore the possible contribution of GAS6 to the adhesive

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[1] The abbreviations used are: ECD, extracellular domain; EGF, epidermal growth factor; HA, hemagglutinin; FACS, fluorescence-activated cell sorter.

function of Axl in mammalian cells. We employed the murine myeloid cell line 32D as a model system to study these effects because, like S2, 32D cells grow in suspension as separate cells. Furthermore, we have shown that 32D cells overexpressing Axl have no mitogenic response to GAS6 treatment and therefore will permit the investigation of nonproliferative GAS6 effects (17). Our work described herein indicates that, unlike insect cells, 32D cells do not form aggregates when Axl is overexpressed. However, GAS6 dramatically induces Axl-mediated cellular aggregation, suggesting that a major role for the Axl-GAS6 interaction is in cell adhesion.

EXPERIMENTAL PROCEDURES

Aggregation Assays—Cells were resuspended in growth medium supplemented with GAS6 and seeded in four consecutive wells of a 24-well plates in a total of 1 ml of medium. Each experiment was repeated three times. Cellular aggregates of at least 200 μm in diameter (the diameter of a single 32D cell is 5 μm) were counted in a single "field of view" using an Olympus model CK 2 microscope at a magnification of $\times$40 with a 10-μm ruler in the eyepiece calibrated with a stage micrometer (Olympus). Five separate fields of view were counted per sample, and the results were averaged. Inhibition assays were conducted by mixing Axl-ECD with GAS6 at a 20:1 or 2:1 molar ratio and incubating this mixture at 37 °C for 30 min before the addition of cells. Bovine serum albumin was purchased from Sigma. Recombinant GAS6 was isolated from transfected Chinese hamster ovary cells grown in medium supplemented with vitamin K as described previously (10). Protein S was purchased from Hematologic Technologies (Essex Junction, VT).

Cell Culture and Cell Lines—Both the 32D-EAK and 32D-Axl cell lines were maintained as described previously (18). Briefly, 32D growth medium includes an exogenous source of interleukin-3 which is supplied by Wee-Hi conditioned medium. Introduction of expression constructs was achieved by infection with recombinant retrovirus in the pLXSN or pLXSP (gift of Dr. John Olson; UNC-Chapel Hill) vector in a manner similar to that of 32D-EAK and 32D-Axl as described previously (18). Briefly, an ecotropic packaging cell line (GP+E86) was transfected by the addition of DNA and LipofectAMINE (Life Technologies, Inc.) and selected with G418 (Life Technologies, Inc.) or 1 μg/ml puromycin (Calbiochem). Retrovirus was harvested from stable cell lines and used to infect 32D cells followed by overnight passage and subsequent selection with either G418 at 500 ng/ml or puromycin at 1 μg/ml.

Recombinant Constructs—The Axl lysine to arginine (32D-Axl K567R) construct was made by cutting the previously described Axl *Sal*I pLXSN vector with *Sal*I and *Xho*I and ligating this to an insert carrying the K567R cytosolic domain derived from the previously described EAK K567R construct by an *Xho*I and *Sal*I digest (18, 19). Positive clones were screened by restriction digests and junctions confirmed by sequencing. The Axl receptor devoid of cytosolic domains (Axl-ECD-TM) was made by digesting the Axl *Sal*I pLXSN vector with *Sal*I and *Xho*I and subsequent ligation of the vector. This creates a truncated Axl gene whose coding sequence stops at 1578 located at the stop insert sequence on the cytosolic side of the transmembrane domain.

Construction of an HA-tagged GAS6 construct was achieved by appending HA to the 3′ end of GAS6. To achieve this, a *Kpn*I site was introduced to the 3′ end of GAS6 by polymerase chain reaction amplification using the pGAS-3 and 3′GAS6-HA oligonucleotides. The sequence of pGAS-3 in 5′-CGAATGCAGCCAGGCCA-3′ and that of 3′GAS6-HA is 5′-CGGGGTACCCGGCTGCGGCGGGCTCCACGG-3′. Following polymerase chain reaction the amplicon was digested with *Bgl*II and *Kpn*I and inserted into the pBS HA (gift of Yue Xiong; UNC-Chapel Hill) vector that had similarly been cut with *Bgl*II and *Kpn*I restriction enzymes. Clones were selected, and the 3′GAS6 segment was shuttled into the pSVSPORT vector. This vector was restricted with *Hin*dIII, *Eco*RI linkers were added, and the construct was subsequently subcloned into pLXSP.

Production of Axl-ECD—The Axl-ECD was made by purifying hexahistidine-tagged Axl-ECD from baculovirus-infected Sf9 cells. Briefly, Sf9 cells (1×10^6 cells/ml with viability greater than 90%) were infected with high titer virus stock and allowed to proceed 5 days until viability was less than 40%. Cells were harvested by centrifugation and cells lysed with Sf9 lysis buffer (1% Nonidet P-40, 300 mM NaCl, 50 mM Na_2HPO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 20 μg/ml phenylmethylsulfonyl fluoride). Lysates were then passed over a Ni^{2+}-NTA slurry (Qiagen) which was washed with 6 M guanidinium HCl, 0.2 M acetic acid, and subsequently equilibrated with 10 mM Na_2HPO_4, pH 7.0, 150 mM NaCl. Bound protein was eluted with 100 mM imidazole and eluate dialyzed to purify protein.

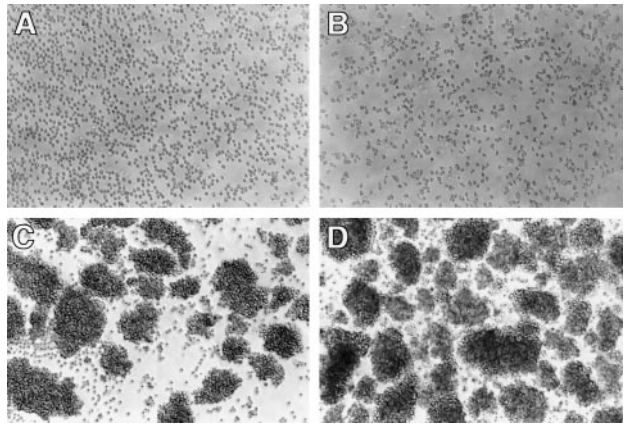

FIG. 1. **GAS6 induces cellular aggregation of 32D-Axl but not 32D-Parental cells.** 32D-Axl cells were treated with GAS6 (220 ng/ml) for 40 h. A picture of the culture was made with a magnification of $\times$ 40. *Panel A*, 32D-Parental cells treated with GAS6. *Panel B*, 32D-Axl cells, untreated. *Panel C*, 32D-Axl cells treated with GAS6. *Panel D*, 32D-Axl-GAS6-HA compound cell line, untreated.

FACS and Western Analysis—An Axl monoclonal antibody directed against the first Ig Loop of Axl (amino acids 33–139) was directly conjugated to phycoerythrin and used to assay Axl surface expression in a Becton Dickinson FACSCAN. Briefly, 800 ng of Axl-phycoerythrin monoclonal antibody was mixed with 1×10^6 cells at 1×10^7 cells/ml and incubated for 20 min on ice. Stained cells were then washed two times with phosphate-buffered saline, 0.1% bovine serum albumin and resuspended at 1×10^6 cells/ml. The reported value represents the mode of fluorescent intensity determined using the flow cytometry software operating package Cyclopes. Surface expression of GAS6 was analyzed with anti-GAS6 antibody at 170 ng/1×10^6 cells followed by immunofluorescence with goat anti-rabbit fluorescent isothiocyanate at 800 ng (Chemicon). Western analysis was conducted on 50 μg of cell lysate using the previously described Axl polyclonal antibodies (18) for Axl expression or phosphotyrosine antibodies for Axl autophosphorylation (Upstate Biotechnology Inc.). Immunoblot analysis for HA-GAS6 was conducted with HA 12-CA5 antibody (Boehringer Mannheim) at 1 μg/ml followed by a goat anti-mouse horseradish peroxidase antibody with the ECL detection system (Amersham Corp.).

Fluorescent Tagging of 32D Cells and Immunofluorescent Microscopy—32D or 32D-GAS6-HA cells were incubated with CellTracker Orange (purchased from Molecular Probes, Eugene, OR) at 0.5 μM for 30 min; 32D-Axl or 32D-Axl-GAS6-HA cells were incubated with CellTracker Green at 10 μM for 30 min (purchased from Molecular Probes). After staining, cells were washed extensively with RPMI and then returned to tissue culture. Green fluorescence was visualized with a band pass filter at 510–560 nM (Nikon Cube G2-A); red fluorescence was analyzed with a band pass filter of 470–490 nM (Nikon Cube B1-E). Unbound GAS6 was washed from cells by a 2-fold resuspension in growth medium and centrifugation. To ensure complete removal of unbound GAS6 the conditioned medium from the ligand-treated and washed cells was assayed for the ability to induce receptor down-regulation of 32D-Axl cells. Two washes were sufficient to remove all unbound GAS6 as assayed by the receptor down-regulation assay (data not shown).

Truncated GAS6 Protein—The deletion of GAS6 truncates sequences amino-terminal to amino acid 279. Conditioned medium from embryonic kidney 293 cells transfected with either empty vector or truncated GAS6 was used as a source of ligand.

RESULTS

GAS6 Induces Cell-to-cell Homotypic Binding of 32D-Axl Cells—The murine myeloid precursor cell line 32D grows as single discrete suspension cells in culture, and 32D-Axl cells exhibit the same growth characteristics as 32D parental cells. Passage of 32D-Axl cells, but not 32D parental cells, in GAS6-supplemented growth medium induces the formation of large cellular aggregates (Fig. 1*C*). Individual aggregates of 200 μm

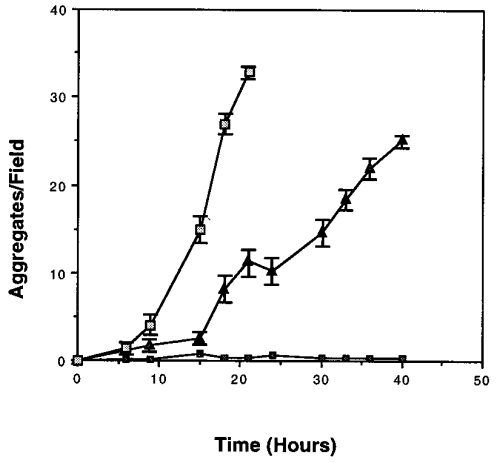

FIG. 2. **Cellular aggregates increase as a function of time.** 32D-Axl, but not 32D-Axl-GAS6, cells were treated with GAS6 (220 ng/ml) and the formation of aggregates monitored as a function of time. Each culture was counted with five separate fields of view, each culture was seeded four different times/experiment, and each experiment was repeated three times. Reported counts are an average of all measurements. Means ± S.E. of four experiments performed in duplicate are shown. ■, untreated; ▲, GAS6-treated; □, 32D-Axl-GAS6-HA.

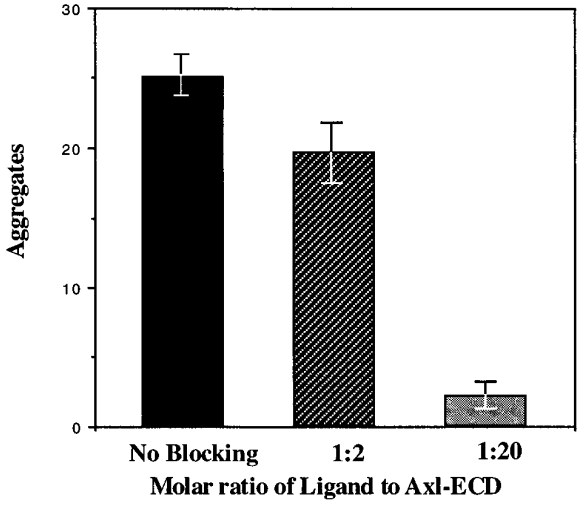

FIG. 3. **GAS6-induced clumping of 32D-Axl cells is inhibited by Axl-ECD.** Cells were seeded at 5×10^5 cells/ml and treated with GAS6 (70 ng/ml) for 40 h in the presence of Axl-ECD. A 1:2 molar ratio of GAS6:Axl-ECD inhibits the clumping by 20%, and a 1:20 ratio inhibits the clumping by 90%. Means ± S.E. of four experiments performed in duplicate are shown.

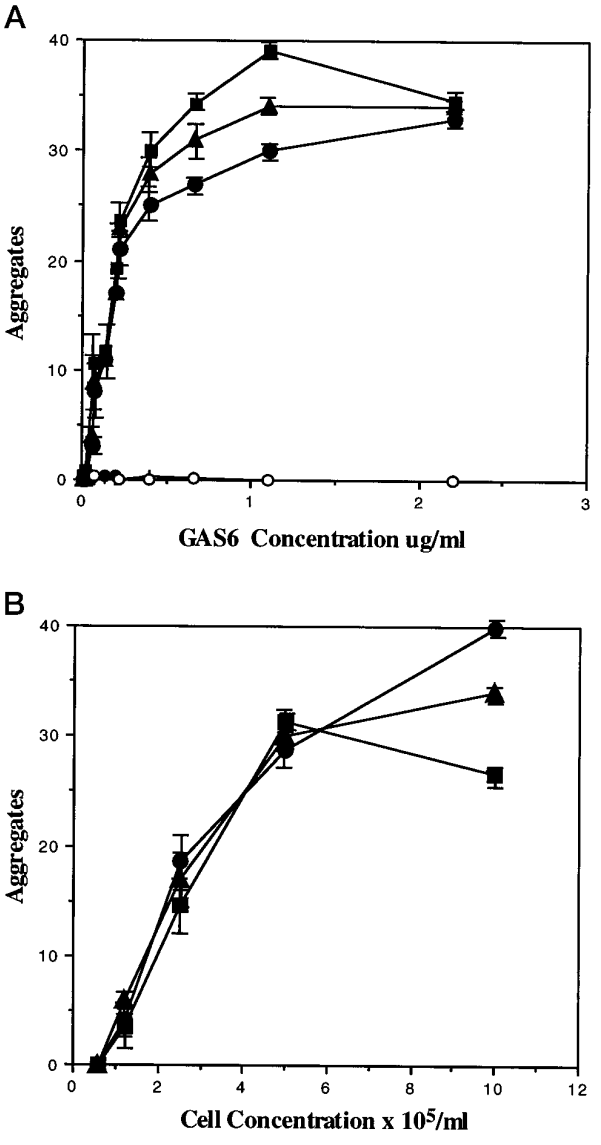

FIG. 4. **Cell and ligand concentration mediate degree of cellular aggregation.** 32D-Axl cells express the wild-type receptor, 32D-Axl K567R cells express a kinase-dead mutant, and the 32D-Axl-ECD cells express an Axl construct devoid of the cytosolic domain. *Panel A*, ligand concentration determines degree of aggregation. Cells were seeded at 5×10^5 cells/ml and treated with GAS6 for 40 h. ■, 32D-Axl; ▲, 32D-Axl K567R; ●, 32D-Axl-ECD; ○, 32D-Parental. *Panel B*, cell concentration determines degree of aggregation. Cultures were treated with 220 ng/ml GAS6 for 40 h. Means ± S.E. of five experiments performed in triplicate are shown. ■, 32D-Axl; ●, 32D-Axl K567R; ▲, 32D-Axl-ECD.

in diameter can be observed as early as 14 h of GAS6 treatment (220 ng/ml) and increase in number as a function of time (Fig. 2). Furthermore, after 40 h of ligand treatment large superaggregates (1.5 mm in diameter) can be detected. At this time point, there is a notable absence of single cells in cultures treated with GAS6 (220 ng/ml for 40 h) with approximately 90% of the cells contained in aggregates (data not shown). Cellular aggregation can be blocked by 90% when soluble Axl-ECD is added in a 20:1 molar ratio of Axl-ECD to ligand and partially blocked (20% blocking) with a 2:1 molar ratio (Fig. 3). That the Axl-ECD effect is specific is supported by the fact that bovine serum albumin was not able to block aggregation when mixed with GAS6 at concentrations similar to that of Axl-ECD. Aggregation is dependent upon the concentration of both ligand and cells, which is consistent with a stoichiometric and saturable process (Fig. 4). This cell-to-cell binding is also blocked by the addition of calcium chelators (5 μM EGTA) as expected for a Gla protein (data not shown). Thus, GAS6 induces aggregation that is specific to Axl-expressing 32D cells and which is dependent on the duration of GAS6 treatment as well as concentration of cells and ligand.

It is possible that the GAS6-induced aggregation of 32D-Axl cells is the result of a contaminant from the isolation of recombinant GAS6. To rule out this possibility, we determined the aggregation profile of a 32D cell line expressing both GAS6 and Axl. An HA-tagged GAS6 recombinant retrovirus was used to infect the 32D parental (to generate 32D-GAS6-HA) and 32D-Axl (to generate 32D-Axl-GAS6-HA) cell lines, and expression of both Axl and GAS6-HA was determined by immunoblot analysis (Fig. 5, *A–C*). Furthermore, the GAS6-HA recombinant protein secreted from these cells is capable of stimulating Axl autophosphorylation, indicating the functional integrity of this protein (Fig. 5*D*). The 32D-Axl-GAS6-HA compound cell

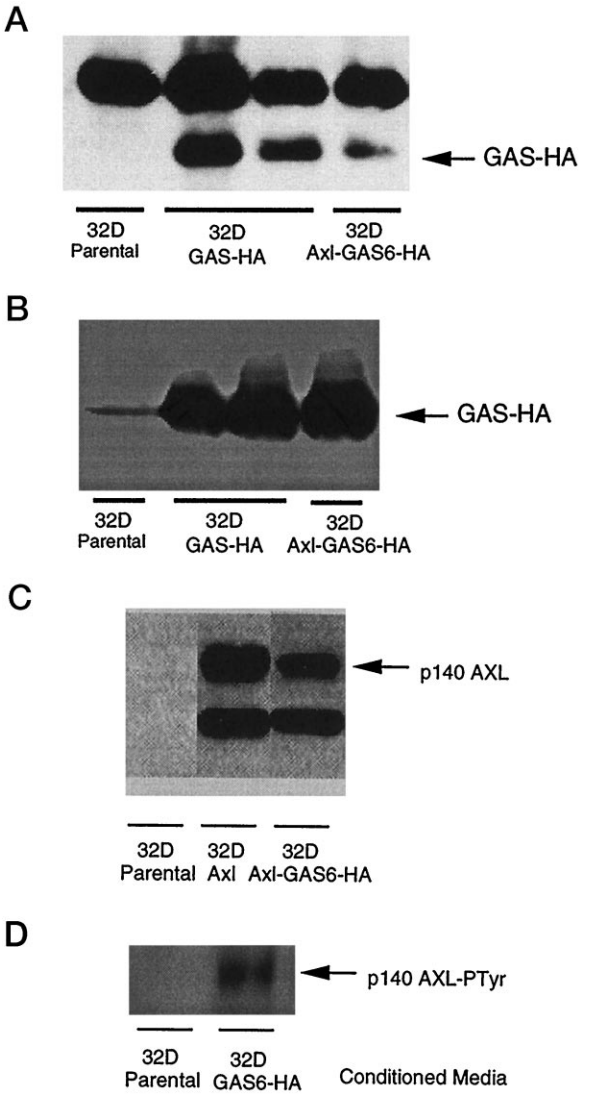

FIG. 5. **Expression and function of GAS6-HA in 32D cells.** 32D cells were infected with a virus carrying a fusion gene of GAS6 with the epitope HA antigen. *Panel A*, conditioned media from various 32D subclones (including 32D-GAS6-HA or 32D-Axl-GAS6-HA cells) was treated with an HA antibody to immunoprecipitate HA-GAS6. The immunoprecipitates were then subjected to Western analysis with the same HA antibody. *Panel B*, cell lysates from the same clones as above were subjected to Western analysis with the HA antibody to determine cellular levels of GAS6. *Panel C*, expression of Axl in the same set of 32D subclones was confirmed by Western analysis with an Axl antibody. *Panel D*, only conditioned medium from 32D-GAS6-HA cells was able to induce Axl phosphorylation in 32D-Axl cells.

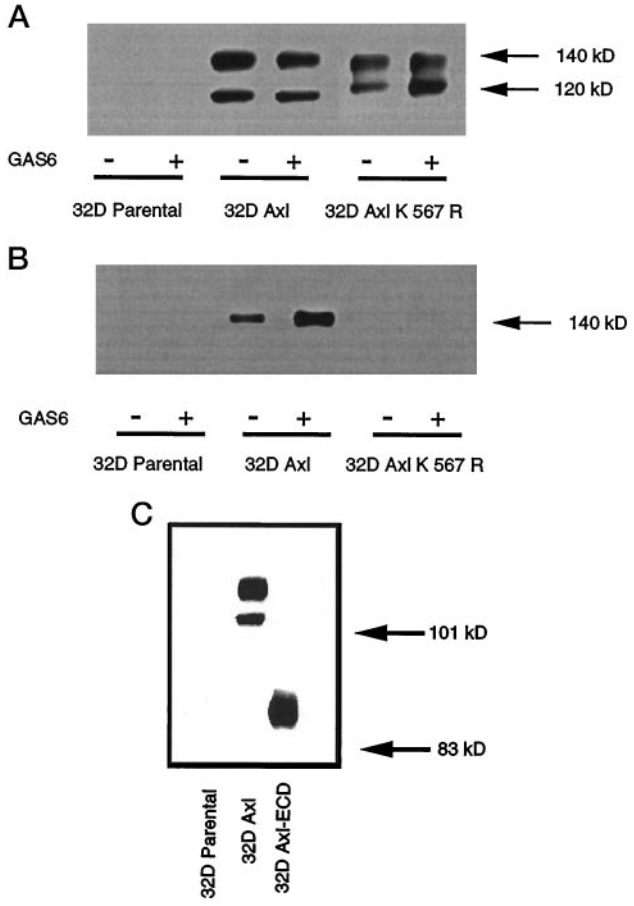

FIG. 6. **Expression of the Axl K567R and Axl-ECD constructs in 32D cells.** 32D-Axl K567R cells express a kinase-dead mutant, and the 32D-Axl-ECD cells express a construct devoid of the cytosolic domain. Expression level of Axl in 32D-Parental, 32D-Axl, 32D-Axl-K567R, and 32D-Axl ECD cells was assayed by Western analysis using an Axl antibody. *Panel A*, Western analysis using the Axl antibody shows that AXL is expressed in either 32D-Axl or 32D-Axl K567R cells regardless of ligand treatment (GAS6, 220 ng/ml for 10 min). *Panel B*, the same set of lysates were immunoprecipated with Axl antibody and subjected to Western analysis with a phosphotyrosine antibody, which shows that cells harboring the Axl K567R mutant, in contrast to 32D-Axl cells, are not able to activate Axl phosphorylation in response to GAS6 treatment. *Panel C*, Western analysis with Axl antibody of either 32D-Parental, 32D-Axl, or 32D-Axl-ECD clones showing the expected sizes for either wild-type p140 Axl or its truncated counterpart p89 Axl-ECD.

line exhibited marked aggregation (Fig. 2) and did not respond further to the exogenous addition of GAS6 (data not shown). In contrast, the 32D-GAS6-HA cell line did not form aggregates. Taken together, these results indicate that GAS6 specifically induces homotypic binding of 32D-Axl cells.

Protein S has a high degree of similarity to GAS6 and, therefore, may be able to induce cellular aggregation in 32D-Axl cells. To test this possibility, 32D-Axl cells were treated with protein S at concentrations comparable to those used for GAS6-induced aggregation (220 and 40 ng/ml). Protein S was not able to mimic the action of GAS6 on cell aggregation at these concentrations (data not shown). This is consistent with previous studies showing that protein S does not bind to Axl in *in vitro* assays (19).

The Kinase Domain of Axl Is Not Required for Aggregation—We have shown previously that treatment of 32D cells expressing an EGF receptor-Axl chimeric receptor tyrosine kinase (32D-EAK) with the foster ligand EGF will result in kinase activation and cellular proliferation (18). However, treatment of 32D-EAK cells with EGF does not result in cell aggregates, suggesting that the Axl-mediated aggregation is independent of the kinase activity (data not shown). To confirm this possibility, we investigated a panel of Axl kinase mutants for their ability to mediate binding. The mutants include a "kinase-dead" Axl whereby the conserved lysine in the ATP binding site was mutated to arginine (32D-Axl K567R) thus inactivating the enzymatic activity of the kinase. Another mutant containing the ECD and plasma membrane-spanning region of the receptor (32D-Axl-ECD) was created by inserting a stop codon at the stop insertion sequence at amino acid 529. Correct size and function of the mutants were confirmed by immunoblot analysis, which showed that the 32D-Axl K567R clone had an Axl antibody-specific band that migrated at 140 kDa and which did not exhibit autophosphorylation in response to GAS6 treatment (Fig. 6, *A* and *B*), whereas the 32D-Axl-ECD clone expressed the expected 89-kDa protein that cross-reacted with the amino-terminal Axl antibody (Fig. 6*C*). Surface ex-

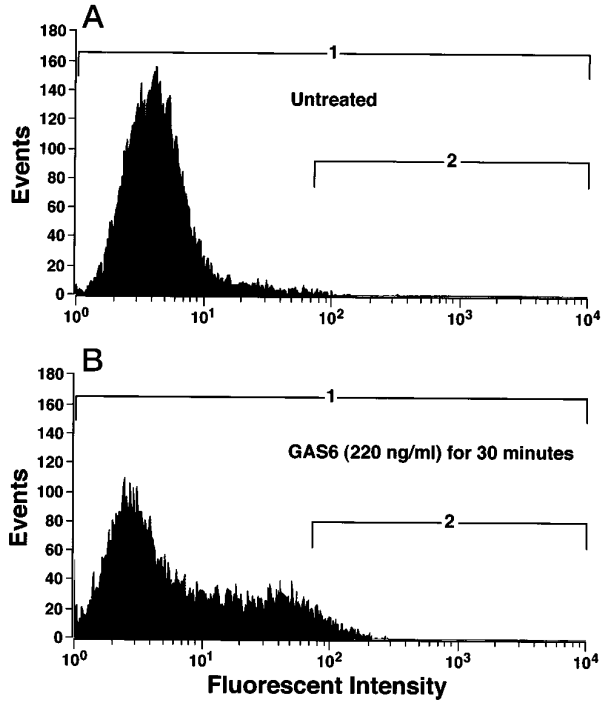

FIG. 7. **GAS6 binds to the surface of 32D cells.** 32D cells were either untreated or treated with GAS6 (220 ng/ml) for 30 min. The amount of surface-bound GAS6 was assayed by staining cells with a rabbit anti-GAS6 antibody and labeled with a fluorescein isothiocyanate goat anti-rabbit antibody. *Panel A* is the fluorescent intensity of untreated cells; *panel B* shows the intensity of GAS6-treated cells.

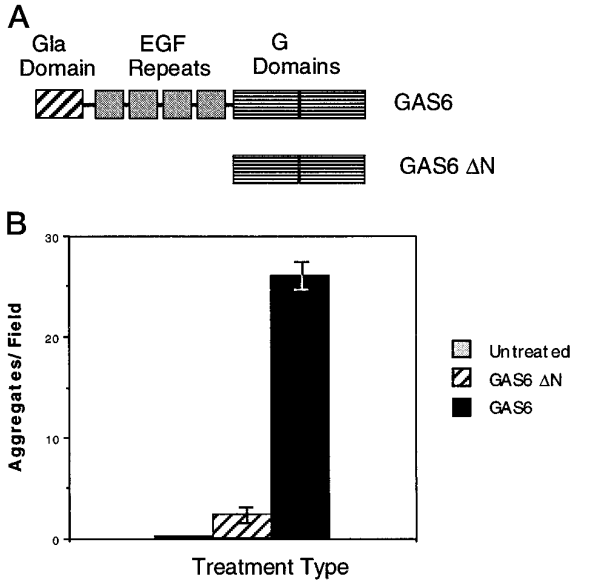

FIG. 8. **Truncated GAS6 does not mediate aggregation.** *Panel A*, structure of GAS6 and the amino-terminal deletion mutant at amino acid 279 which retains Axl binding activity. *Panel B*, 32D-Axl cells were treated with full-length GAS6 or amino-terminal truncated GAS6 and the aggregation profile characterized. *Light column*, untreated; *striped column*, GAS6ΔN; *dark column*, GAS6.

pression of mutants was confirmed by FACS analysis, which showed that both the 32D-Axl K567R and the 32D-Axl-ECD cultures contain about half the amount of surface receptor as did the 32D-Axl cultures (data not shown). However, when tested for their ability to induce cellular aggregation in response to GAS6, both 32D-Axl K567R and 32D-Axl-ECD clones were capable of mediating cellular aggregation at a level equal to that of 32D-Axl cells (Fig. 4). Furthermore, the kinetics of aggregation for the two mutant receptors closely matched that of the wild-type Axl receptor. Thus, the ability of GAS6 to induce homotypic binding is mediated solely by the transmembrane and extracellular domains of Axl and is independent of the kinase function.

GAS6 Binds to 32D Cells and Renders Them Capable of Binding to 32D-Axl Cells—One mechanism to bring about aggregation includes the binding of GAS6 to the surface of one cell and simultaneous interaction with Axl receptor on an adjacent cell. To investigate this possibility we treated 32D cells with GAS6 (220 ng/ml) and analyzed the amount of GAS6 which was cell surface-bound. Surface-bound GAS6 protein was measured by flow cytometric analysis of cells labeled with a rabbit anti-GAS6 antibody followed by goat anti-rabbit fluorescein isothiocyanate and could be observed as early as 30 min (Fig. 7) after the addition of GAS6 and as late as 27 h (data not shown), indicating that GAS6 remains bound during the time required to form aggregates. Cell surface binding was shown to be dependent on divalent cations as 5 μM EGTA blocked surface display of GAS6 (data not shown). This indicates that surface binding of GAS6 may be an important component of aggregation mechanics. In support of this is the enhanced aggregation observed in a 32D-Axl-GAS6-HA cell line that has high levels of Axl expression and also expressed GAS6 that was primarily membrane-bound.[2] This indicates that surface-bound GAS6 can be a primary determinant in mediating adhesion of Axl-expressing cells.

We mapped the motifs in GAS6 which mediate adhesion by treating 32D-Axl cells with a truncated form of GAS6. The deletion is amino-terminal to the first G domain (Fig. 8*A*) and deletes the EGF-like repeats as well as the Gla domain at amino acid 279, leaving only the two G domains. Treatment of 32D-Axl cells with medium containing the truncated GAS6 ligand did not mediate aggregation (Fig. 8*B*) despite its ability to induce phosphorylation of the Axl receptor.[3] This study indicates that either the Gla domain and/or the EGF motifs are necessary for GAS6-mediated aggregation of 32D-Axl cells.

If GAS6 binds to the surface of 32D cells, then, in our proposed model, 32D-Axl cells should be able to form mixed cell aggregates with GAS6-bound 32D cells. To test this possibility we treated 32D-GAS6-HA cells with a rhodamine-labeled fluorescent dye (CellTracker Orange). This lipophilic label traverses the cell membrane and is cleaved by intracellular proteases, which results in the covalent binding of the label to intracellular constituents. This permits the tracking of a unique population of cells by the assessment of red fluorescence. 32D-Axl cells were treated similarly with eosin diacetate (CellTracker Green), which labels cells green. When these two populations were cocultured, we found that 100% of the aggregates contained both cell populations (Fig. 9 and Table I). As before, the coincubation of 32D with 32D-Axl or 32D-GAS6-HA cells did not induce cell aggregates. These data support the notion that Axl and GAS6 induce cell adhesion through a heterotypic intercellular mechanism. 32D-GAS6-HA cells express high levels of surface GAS6 which may potentially alter membrane charge characteristics to support mixed cell aggregation. To assess this possibility, we incubated 32D parental cells with GAS6 for 30 min followed by washing of unbound ligand. This results in a significantly lower amount of surface bound GAS6 than when the GAS6 cDNA is transfected into 32D cells. These cells were cocultured with 32D-Axl cells and were found to induce 100% mixed aggregates, suggesting that

[2] Y. W. Fridell, unpublished results.

[3] B. Varnum, unpublished results.

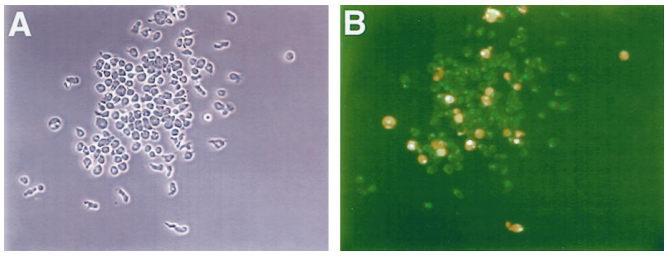

FIG. 9. **Formation of mixed cell aggregates.** The 32D-GAS6-HA cell line was treated with a rhodamine (*red*) fluorescent dye that traverses the cell membrane and then becomes immobilized in the cytoplasm. 32D-Axl cells were treated with BODIPY (*green*) fluorescent dye that is immobilized in the cytoplasm in a similar fashion. The two cell types were then mixed, and the differentially labeled cells present in the aggregates were characterized after 24 h of coculture. The aggregates were transferred by pipette to glass slides and coverslips placed on the cell solution. Photographs were made either in bright field (*panel A*) or with a double exposure (*panel B*) using fluorescent microscopy with a band pass filter, which allowed visualization of the appropriate marker while excluding the alternate marker. No autofluorescence of the cells was observed. These results indicate the presence of 32D-GAS6-HA cells mixed with 32D-Axl cells. This suggests that GAS6, bound to the surface of 32D-Parental cells, may permit heterotypic interaction with Axl-expressing 32D cells.

TABLE I

Composition of aggregates in various culture conditions

Cells were either untreated or treated with GAS6 (220 ng/ml). In cases where cells were washed of GAS6, the ligand exposure period was for 30 min. The results are presented as a proportion of the aggregates containing both cell types (mixed aggregates).

Types of cells mixed		Treatment with GAS6	Washing	% Mixed aggregates
I	II			
32D-GAS6-HA	32D-Axl	None	None	100
32D	32D-Axl	I and II	None	100
32D	32D-Axl	I only	Yes	100
32D	32D-Axl	II only	Yes	26
32D	32D-Axl	None	None	0

the GAS6-Axl interaction is not dependent on high levels of local GAS6 expression.

To eliminate the possibility that GAS6 induces aggregation only of 32D-Axl cells and that the formation of mixed aggregates is the result of nonspecific recruitment during the adhesion process, untreated 32D parental cells were coincubated with 32D-Axl cells that were pretreated with exogenous GAS6. The predicted interaction should be between pretreated 32D-Axl cells only and would exclude the untreated 32D parental cells. The proportion of mixed cell aggregates would be therefore a measure of background aggregation. In this experiment, only 26% of the cellular aggregates were of a mixed cell type compared with 100% in all other combinations. Therefore, our data confirm that membrane-bound GAS6 is able to mediate heterotypic interactions with Axl-expressing cells.

DISCUSSION

GAS6 has several structural motifs, which suggests that this polypeptide can participate in combinatorial interactions (11, 12). Consistent with this prediction we have provided evidence that GAS6 has at least two distinct functions. We have shown that GAS6 binding to the cell surface is dependent on the Gla and EGF domains in the presence of Ca^{2+}, whereas the Axl activation activity is dependent only on the steroid hormone binding domains (G domains). This is consistent with the observation that the Gla domain of protein S, a GAS6 homolog, interacts with negatively charged phospholipids, and the EGF repeats interact with integral membrane proteins in a calcium-dependent fashion (14, 15). Thus, these domains may permit targeting of GAS6 to the plasma membrane while the G domains interact directly with Axl receptor. As the kinase activity of Axl is dispensable for this interaction it appears that Axl-mediated aggregation does not require interaction of downstream signaling pathways. This contrasts with suggestions of earlier reports where high levels of Axl expression in insect cells induced cellular aggregation that correlated with a slight increase in the level of Axl phosphorylation (6). Nevertheless, when taken together, our data indicate that one biological outcome of the GAS6-Axl interaction is to bring cells into close apposition.

Stimulation of endogenous Axl with the native ligand does not result in a growth response except in a limited number of cell lines (17). This brings into question whether the primary function of Axl is mitogenic. That proliferative responses may be a limited aspect of receptor tyrosine kinase signaling is best demonstrated by the ability of the Eph family to mediate fasciculation and collapse of axons of the retinal ganglion to the optic tectum but not mitogenesis. In this manner, the ligand-receptor interaction regulates a structural determinant such as topographical targeting of axons (20). Thus, failure of GAS6 to drive proliferation of Axl-expressing cells supports the notion that a physiologic role of this ligand receptor interaction may involve nonmitogenic processes.

Previous studies of Axl expression in hematopoiesis have shown that Axl is expressed predominantly in the bone marrow stroma, and increased expression is observed upon stimulation of peripheral blood with phorbol esters or interferon-α (21). In addition, phenotypic analysis of bone marrow-derived cells shows that mature monocytes ($CD14^+$) express Axl.[4] Several investigations have shown that interferon-α treatment of monocytes leads to increased adhesion, suggesting that Axl may participate as a cell adhesion receptor in activated monocytes (22–24). In development, Axl is expressed in mesenchymal cells during organogenesis,[5] raising the possibility that coordinated expression of Axl and GAS6 may also regulate some aspects of cell migration during embryogenesis. Taken together, our observations suggest that a potentially important role of the Axl-GAS6 interaction is in cell adhesion, particularly when involved with hematopoiesis and development.

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