Title

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Journal

Molecular Biology of the Cell, 10(2)

ISSN

1059-1524

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Publication Date

1999-02-01

α1 and α2 Integrins Mediate Invasive Activity of Mouse Mammary Carcinoma Cells through Regulation of Stromelysin-1 Expression

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LBNL/DOE funding & contract number: DE-AC02-05CH11231

Tumor cell invasion relies on cell migration and extracellular matrix proteolysis. We investigated the contribution of different integrins to the invasive activity of mouse mammary carcinoma cells. Antibodies against integrin subunits α6 and β1, but not against α1 and α2, inhibited cell locomotion on a reconstituted basement membrane in two-dimensional cell migration assays, whereas antibodies against β1, but not against a6 or α2, interfered with cell adhesion to basement membrane constituents. Blocking antibodies against α1 integrins impaired only cell adhesion to type IV collagen. Antibodies against α1, α2, α6, and β1, but not α5, integrin subunits reduced invasion of a reconstituted basement membrane. Integrins α1 and α2, which contributed only marginally to motility and adhesion, regulated proteinase production. Antibodies against α1 and α2, but not α6 and β1, integrin subunits inhibited both transcription and protein expression of the matrix metalloproteinase stromelysin-1. Inhibition of tumor cell invasion by antibodies against α1 and α2 was reversed by addition of recombinant stromelysin-1. In contrast, stromelysin-1 could not rescue invasion inhibited by anti-α6 antibodies. Our data indicate that α1 and α2 integrins confer invasive behavior by regulating stromelysin-1 expression, whereas α6 integrins regulate cell motility. These results provide new insights into the specific functions of integrins during tumor cell invasion.

Introduction

Invasion of basement membranes and of stromal extracellular matrix (ECM) is a rate-limiting step for establishment of tumor metastases. Invasive behavior is a multistep process consisting of adhesion to, proteolysis of, and migration along ECM (Liotta *et al.*, 1983). Although adhesion to ECM is an a priori requirement for ECM invasion, cell migration along the ECM involves the dynamic establishment and dissolution of cell–ECM contacts, i.e. adhesion and deadhesion. One critical parameter that determines the rate of cell migration is the degree of adhesiveness of cells to their ECM substrata. This has been corroborated by mathematical models (DiMilla *et al.*, 1991), as well as by experimental evidence (Goodman *et al.*, 1989; Halfter *et al.*, 1989; Duband *et al.*, 1991; DiMilla *et al.*, 1993), showing that intermediate levels of cell-to-substratum adhesion are required for maximal cell migration. Thus, the composition of ECM and the repertoire of ECM receptors on the cell surface are intimately involved n the regulation of these processes (Damsky and Werb, 1992; Heino, 1996).

Integrins, the major and best characterized group of ECM receptors, have attracted considerable attention in the effort to unravel the mechanism of cell migration and/or invasion. In mammals, currently 16 different integrin a and 8 different integrin b subunits are known (Giancotti, 1997). In most cell types, integrins appear to be essential for cell adhesion to individual ECM constituents (Hynes, 1992). However, because cells in vivo interact with an ECM of complex composition as well as with other cells, the participation of individual integrins in adhesive events cannot be studied easily under such conditions. Furthermore, nonintegrin ECM receptors, such as cell surface proteoglycans or immunoglobulin (Ig) superfamily adhesion molecules, also play a role in cell adhesion to the ECM (Zisch *et al.*, 1992; Mercurio, 1995; Noat *et al.*, 1997; Powell and Kleinman, 1997).

Although some studies show expression of certain integrin subunits as positively correlated with invasion (Cannistra *et al.*, 1995; Melchiori *et al.*, 1995; Chao *et al.*, 1996; Matsuura *et al.*, 1996; Vihinen *et al.*, 1996; Trikha *et al.*, 1997), others show that integrins can diminish invasion (Damsky *et al.*, 1994; Paulus and Tonn, 1994; Danen *et al.*, 1996). One well characterized mechanism by which integrins affect tumor cell invasion is the establishment of short-lived adhesive contacts with the ECM (DiMilla *et al.*, 1991; Duband *et al.*, 1991; Palecek *et al.*, 1997), a process that is accompanied by rapid changes in cytoskeletal microarchitecture, which are prerequisite for cell motility (Kassner *et al.*, 1995; Lauffenburger and Horwitz, 1996). Because integrins can trigger diverse signaling events that lead to alterations in gene expression (Schwartz *et al.*, 1995; Kheradmand *et al.*, 1998), modulation of synthesis of other proteins that are important for invasion, including ECM-degrading enzymes (Heino, 1996), may be a critical event regulating tumor cell invasion. Integrins also facilitate invasion by recruiting ECM-degrading proteinases to sites where proteolysis is required (Brooks *et al.*, 1996).

In this study we used function-blocking antibodies against α1, α2, α5, α6, αv, and β1 integrins to dissect their specific functions in cell invasion, migration, adhesion, and matrix metalloproteinase production. We show that different integrins mediate these processes via distinct mechanisms in mammary epithelial cells.

Materials and Methods

Recombinant Stromelysin-1 and Antibodies

Human stromelysin-1 with the C-terminal hemopexin domain truncated was expressed and purified from the methyltrophic yeast *Pichia pastoris* (Smith, Sharkov, and Navre, unpublished results). In brief, a portion of the human stromelysin-1 cDNA (from the start of the prodomain to the hinge region: PLDGAA to PDSPET) (Whitham *et al.*, 1986) was inserted behind the α factor signal sequence in the vector pPIC9 (Invitrogen, San Diego, CA). The resultant plasmid was transformed into *Pichia pastoris*, and eight methanol use-deficient clones were screened for production of recombinant stromelysin-1. One overproducing clone was selected and used for all expression and purification work. Induction of an AOX1 promoter was accomplished using the *Pichia* Expression Kit (Invitrogen), according to the manufacturer's instructions. The cell culture medium containing the recombinant stromelysin-1 was cleared of cells by centrifugation and dialyzed against two to three changes of 0.02% Brij-35 in 20 mM HEPES buffer, pH 7.0. The dialysate was loaded on a 100-ml column of reactive red agarose (Sigma, St. Louis, MO) that was equilibrated with HCB buffer (0.02% Brij-35 and 5 mM $CaCl_2$ in 20 mM HEPES, pH 7.0). After the column was washed with HCB buffer, the protein was eluted using a 160-ml 0–2 M NaCl gradient in HCB buffer. For further purification, the eluted protein was dialyzed against HCB buffer, bound to a 5-ml column of Q-Sepharose Fast Flow (Pharmacia, Piscataway, NJ), and eluted with 160 ml of a 0–1 M NaCl gradient in HCB buffer. Mass spectrometry and N-terminal sequencing of the final product were consistent with the protein being intact and with no apparent modifications, such as glycosylation. Casein zymography indicated that the recombinant stromelysin-1 was proteolytically active. Before use in cell culture experiments, stromelysin-1 was dialyzed

against DMEM/F12 (Life Technologies, Gaithersburg, MD). Stromelysin-1 at a concentration of 1 μg/ml was activated by incubation with trypsin (Life Technologies) at 1 mg/ml for 1 h at 37°C. Trypsin was subsequently inhibited with soybean trypsin inhibitor (Sigma) added at a final concentration of 10 μg/ml.

All function-blocking monoclonal antibodies against integrin subunits were obtained from PharMingen (San Diego, CA). Azide-free preparations of antibodies against α1 (clone Ha31/8), α5 (clone 1A29), αv (clone H9.2B8), and β1 (clone Ha2/5) were purchased. Custom-made azide-free preparations of α6 antibodies (clone GoH3) were purchased. Azide-containing preparations of α2 antibodies (clone HMα2) were purchased and used with similar results either before or after dialysis against phosphate-buffered saline, pH 7.4 (PBS).

Promoter Constructs

A genomic DNA clone containing the mouse stromelysin-1 gene identified in a SuperCos I (Stratagene, La Jolla, CA) library (gift from Dr. John S. Mudgett, Merck Research Laboratories, Rahway, NJ) was used to isolate an *Eco*RI-*Pst*I genomic fragment encompassing ~1.3 kb of 5′-untranslated sequences and ~0.2 kb of the first exon of stromelysin-1. The *Eco*RI-*Pst*I fragment was subcloned into Bluescript KS (Stratagene) and sequenced with the CircumVent sequencing kit (New England BioLabs, Beverly, MA) according to the manufacturer's instructions. The 5′-untranslated sequences were then amplified by PCR and subcloned into the *Kpn*I-*Bgl*II cloning sites of the pGL2 vector (Promega, Madison, WI) upstream from a luciferase reporter gene. The control vector containing the Rous sarcoma virus (RSV) promoter attached to a β-galactosidase reporter gene has been described (Li *et al.*, 1992).

Cell Culture and Transfection

The mouse mammary carcinoma cell line SCg6 (Desprez *et al.*, 1993; Lochter *et al.*, 1997b) was maintained and passaged routinely in medium containing 5% fetal bovine serum, 5 μg/ml insulin (Sigma), and 50 μg/ml gentamycin (Life Technologies) as described (Desprez *et al.*, 1993; Lochter *et al.*, 1997b). All assays were performed in chemically defined medium consisting of DMEM/F12, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium (added as ITS medium supplement; Sigma), and 50 μg/ml gentamycin.

SCg6 cells were transfected with promoter constructs by the use of the Lipofectin reagent (Life Technologies) and pools of stable clones selected as described (Lochter *et al.*, 1997a). In brief, 1.2 X 10^6 cells were maintained in culture dishes 10 cm in diameter (Falcon; Becton Dickinson, Franklin Lakes, NJ) and incubated for 24 h with 5 ml of OptiMEM (Life Technologies) containing 40 μl of Lipofectin, 0.5 μg of SV40neo (Schmidhauser *et al.*, 1992), 3.75 μg of stromelysin-1 promoter construct in pGL2, and 3.75 μg of RSV–β-galactosidase vector. Subsequently, the medium was replaced with medium containing serum. Two days after transfection, cells were selected by addition of 200 μg/ml Geneticin (Life Technologies) to the culture medium. Surviving cells,

originating from ~200 surviving clones per dish, were pooled and expanded in medium containing serum.

Zymograms and Immunoprecipitations

For zymography of proteinases in conditioned medium, 10^6 cells were plated in 1 ml of chemically defined medium into dishes 3.5 cm in diameter (Falcon). Medium was collected 2 d later, and gelatin and casein zymography was performed as described (Fisher and Werb, 1995; Lochter *et al.*, 1997b). To detect gelatinases and caseinases, 2 and 20 µl, respectively, of conditioned medium was loaded per lane.

Immunoprecipitations on cell lysates were performed as described (Lochter *et al.*, 1997a). In brief, cells were metabolically labeled for 16 h with 200 µCi of ^{35}S-methionine (Amersham, Arlington Heights, IL) per milliliter of culture medium. Radiolabeled cells were washed with chemically defined medium and lysed in Nonidet P-40 (NP-40) lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 1% NP-40). Antibodies to integrin subunits were added to cell lysates at a final concentration of 10 µg/ml and were incubated overnight at 4°C. Protein G agarose (Sigma) was then added, and incubation was performed for 1 h at 4°C. Protein G agarose beads were washed with NP-40 lysis buffer and subsequently with 50 mM Tris, pH 7.5. Immunoprecipitates were separated on 5% SDS-polyacrylamide gels under nonreducing conditions. Gels were then dried and processed for autoradiography.

Histochemistry

To analyze the morphology of cells maintained on reconstituted basement membrane (rBM; Matrigel; Collaborative Research, Bedford, MA) gels in the presence of 10 µg/ml anti-integrin antibodies, we fixed cells with 5% glutaraldehyde in PBS and stained the cells with toluidine blue O (Sigma) as described (Lochter *et al.*, 1991). rBM gels were prepared according to published procedures (Lochter *et al.*, 1997b).

Dimethylthiazolyl-diphenyltetrazolium Bromide (MTT) Assay

To determine the number of viable cells, we added 20 µl of 5 mg/ml MTT (Sigma) in PBS to 100 µl of the culture medium of cells maintained for 2 d on rBM gels in 96-well tissue culture plates (Falcon) in the presence of 10 µg/ml antibodies against integrins. Cells were incubated for 1 h at 37°C, whereupon the culture medium was removed and the metabolized MTT was solubilized with 50 µl of dimethylsulfoxide. Absorbance was measured at 570 nm.

Assays for Luciferase and b-Galactosidase Activity

To measure the activity of the luciferase reporter gene, we plated cells at a density of 10,000 cells per well in 96-well multiwell plates. Cells were maintained on untreated tissue culture plastic, on substrata coated with rBM gels, or in suspension culture in which cell adhesion was blocked by coating wells with 2 mg/ml poly(2-hydroxyethyl

methacrylae) (poly-HEMA; Sigma) as described (Roskelley *et al.*, 1994). Antibodies against integrins were added at the concentrations indicated (see Figure 8). As determined by the MTT assay, the number of viable cells was unaffected by the highest concentration of each antibody used (our unpublished results). After 2 d in culture, cells were lysed with 1 mM dithiothreitol and 1% (wt/vol) Triton X-100 in 100 mM sodium phosphate buffer, pH 7.5. Luciferase activity was assayed in a luminometer (Wallac, Gaithersburg, MD) after addition of an equal volume of 150 μg/ml beetle luciferin (Promega), 9 mM ATP, 20 mM $MgCl_2$, 10 mM potassium phosphate, pH 7.5, and 50 mM HEPES, pH 7.4. Activity of β-galactosidase was analyzed with a luminometer with the Galacto-Light Plus kit (Tropix, Bedford, MA), according to the manufacturer's instructions.

Cell Adhesion Assays

For adhesion assays, 96-well tissue culture plates were coated for 2 h at 37°C with laminin (purified from Engelbreth-Holm-Swarm sarcomas; Sigma), type I collagen (Collagen, Fremont, CA), type IV collagen (Collaborative Research), or fibronectin (Collaborative Research), all at 20 μg/ml in PBS. Wells were then washed twice with PBS, and nonspecific binding sites were blocked by incubation with 2 mg/ml heat-inactivated, fatty acid-free bovine serum albumin (BSA; Sigma) in PBS for 1 h at 37°C. Heat inactivation was achieved by incubation of BSA in PBS for 6 min at 80°C. After blocking, wells were washed twice with PBS before addition of 50 μl of chemically defined medium containing antibodies at twice the final concentrations (see Figure 4). Subsequently, 20,000 cells in 50 μl of chemically defined medium were added per well and allowed to adhere for 1 h at 37°C. Adhesion assays were terminated by the addition of 100 μl per well of 5% glutaraldehyde in PBS to the cell culture medium. Cells were fixed for 15 min at ambient temperature, washed three times with water, and stained for 1 h with 1 mg/ml crystal violet (Sigma) in water (Gillies *et al.*, 1986). Finally, cells were washed five times with water and incubated for 1 h with 0.3% (wt/vol) Triton X-100 in water to solubilize crystal violet. Absorbance was measured at 600 nm.

Cell Migration and Invasion Assays

Cell migration assays were performed in modified Boyden chambers with polyterephtalate filter inserts for 24-well plates containing 8-μm pores (Collaborative Research). Both the bottom and the top of the filters were coated with 20 μg/ml laminin, 20 μg/ml fibronectin, or 100 μg/ml rBM in PBS by incubation for 2 h at 37°C. Subsequently, chambers were washed twice with PBS and incubated for 1 h with heat-inactivated fatty acid-free BSA. After two washes with PBS, the lower chamber was filled with 300 μl of medium, and 20,000 cells were plated in each upper chamber in 200 μl of medium. Immediately after cell plating, antibodies (10 μg/ml final concentration), the matrix metalloproteinase inhibitor GM6001 (10 μM), its inactive structural homologue GM1210 (10 μM) (gifts from Dr. R.Galardy, Glycomed Corporation, Alameda, CA) (Grobelny *et al.*, 1992), or activated recombinant stromelysin-1 (1 μg/ml) was added. Migration assays were terminated 6 h after cell plating by fixation with 5%

glutaraldehyde in PBS. Cells that had not migrated to the bottom of the filters were then removed, and the remaining cells were stained with toluidine blue and counted as described (Lochter *et al.*, 1997b).

Invasion assays were performed in the same type of modified Boyden chambers in which the migration assays were performed and according to published procedures (Lochter *et al.*, 1997b); 100,000 cells per well were plated on top of filters coated with 10 μl of rBM gels. After an incubation period of 24 h, cells were fixed and analyzed as described for the migration assay. All antibodies used in invasion assays were applied at a final concentration of 10 μg/ml immediately after cell plating. Recombinant stromelysin-1 was used at a final concentration of 1 μg/ml.

RESULTS

Integrin Subunits a1, a2, a6, and b1 Are Involved in rBM Invasion by Mammary Carcinoma Cells

To study the participation of integrins in tumor cell invasion, we used the highly aggressive mouse mammary carcinoma cell line SCg6 (Lochter *et al.*, 1997b). In culture, SCg6 cells readily invade a rBM (Lochter *et al.*, 1997b). SCg6 cells expressed integrin subunits α1, α2, α5, α6, and β1 but not αv (Figure 1). Antibodies against integrin subunits α1, α2, α5, and α6 coimmunoprecipitated a band corresponding to the molecular weight of the β1 integrin subunit (Figure 1). The β4 integrin subunit was not found in immunoprecipitations with antibodies against the α6 integrin subunit (Figure 1), indicating that α6β1, not α6β4, was the predominant α6-containing integrin expressed by SCg6 cells. This is in contrast to nonmalignant mouse mammary epithelial cells that express the α6β4, but not the α6β1, integrin (Delcommenne and Streuli, 1995; Lochter *et al.*, 1997a).

Antibodies against α1, α2, and β1, but not α5 or αv or control IgGs, at 50 μg/ml (our unpublished results) or 10 μg/ml (Figure 2) abolished the invasion of SCg6 cells in modified Boyden chambers that were coated with rBM, whereas antibodies against α6 integrins reduced tumor cell invasion by one-half. There was no difference in plating efficiency or survival between the inhibitory and noninhibitory treatments (Figure 3A). However, antibodies against β1 integrins, but not any of the other antibodies tested, altered the morphology of cells plated on rBM. Whereas SCg6 cells on rBM formed a network of interconnected and elongated cell aggregates from which invasion occurred at the periphery of cell clusters, cells treated with anti-β1 antibodies appeared to be primarily single and barely spread (Figure 3, B–F). The latter observation indicates that cell–rBM interactions are affected by antibodies against β1.

β1 Integrins Are Used for Adhesion of SCg6 Cells to Basement Membrane Constituents

To analyze whether the integrins involved in rBM invasion by SCg6 cells are used to mediate cell adhesion to major basement membrane constituents, we subjected

cells to adhesion assays in the presence of the integrinblocking antibodies that interfered with rBM invasion. Within 1 h after plating, .90% of cells adhered to substrata coated with laminin, type IV collagen, fibronectin, and type I collagen (our unpublished results). Antibodies against β1 integrins prevented cell adhesion to laminin and type I collagen and reduced adhesion to type IV collagen and fibronectin (Figure 4). Antibodies against a1 integrins reduced cell adhesion to type I and IV collagens at 10 μg/ml and to laminin slightly at 50 and 100 μg/ml (Figure 4). In contrast, antibodies against α2 and α6 integrins alone (Figure 4) and in combination (our unpublished results) had little effect on SCg6 cell adhesion. Therefore, none of the α subunits tested is a major laminin receptor, whereas α1 integrins expressed by SCg6 cells appear to be the major adhesion receptor for type I collagen. These results raise the question of how α1, α2, and α6 integrins inhibit rBM invasion.

Integrin α6 and β1 Subunits Regulate Cell Motility

One possible mechanism by which integrins α1, α2, and α6 could regulate tumor cell invasion may be by interfering with cell motility. In cell migration assays performed using modified Boyden chambers coated with soluble rBM material to yield two-dimensional substrata (2D rBM), as opposed to the three-dimensional rBM gels used for the invasion assays, cell migration was almost completely inhibited by antibodies against β1 integrins (Figure 5A), and antibodies against α6 integrins inhibited SCg6 cell migration to an extent similar to that seen in rBM invasion (;50%; Figure 5A). Anti-β1 and -α6 antibodies gave similar results when laminin, instead of rBM, was used as a substratum (Figure 5B). Antibodies against α1, α2, α5, or αv integrins or control IgGs had no significant effect on SCg6 cell migration on 2D rBM or laminin (Figure 5, A and B). Migration of SCg6 cells on fibronectin was not affected by anti-α6 antibodies (Figure 5C). Thus, α6 integrins appear to contribute to rBM invasion by affecting the ability of SCg6 cells to migrate.

Integrin Subunits α1 and β2 Mediate Invasion by Regulating Proteinase Expression

The results obtained thus far suggest that the mechanism by which α1 and α2 integrins contribute to SCg6 cell invasion is different from that of α6 and other β1 integrins, because neither cell attachment or adhesion to rBM nor cell migration was markedly affected by antibodies against these integrins. ECM-degrading proteinases are required for basement membrane invasion by tumor cells (MacDougall and Matrisian, 1995) and are regulated by integrins in some cell types (Heino, 1996). We therefore analyzed whether the secretion of proteinases by SCg6 cells was affected by antibodies against α1 and α2 integrins. SCg6 cells secreted the proenzyme form of gelatinase B, latent and active isoforms of gelatinase A, stromelysin-1, and an unidentified matrix metalloproteinase migrating at 80 kDa in casein substrate gels (Lochter *et al.*, 1997b) (Figure 6). Antibodies against α2 and α6 integrins, but not against α1 or β1 integrins, reduced expression of both gelatinases slightly (Figure 6). Expression of stromelysin-1 activity was unaffected by antibodies against α6 and β1 integrins but was strongly inhibited by antibodies against α1 and α2 integrins (Figure 6). Expression of the 80-kDa caseinase was increased by antibodies against integrin subunits α1, α2, and β1 (Figure 6).

These data are further evidence that the antibody effects were specific rather than attributable to toxicity of the antibody preparations.

Stromelysin-1 is required for the invasion of rBM by SCg6 cells (Lochter *et al.*, 1997b). Therefore we hypothesized that antibodies against α1 and α2 integrins interfere with rBM invasion by SCg6 cells by impairing stromelysin-1 expression. To test this hypothesis, we reconstituted the system by adding back recombinant stromelysin-1. Addition of stromelysin-1 to the culture medium overcame the block in invasion induced by anti-α1 and -α2 antibodies (Figure 7A) but did not affect SCg6 cell invasion in the presence of antibodies against α6 or β1 integrins (Figure 7B). These data support the concept that α6 and other β1-containing integrins inhibit tumor cell invasion by interfering with the ability of cells to interact with and/or migrate along rBM. When recombinant stromelysin-1 was added to cultures not treated with antibodies, invasion was increased approximately threefold (288 ± 53.6% of control). This observation supports the notion that increased stromelysin-1 activity correlates with increased invasive activity of SCg6 cells, as long as α6 and β1 integrin functions are not compromised.

SCg6 cells did not use stromelysin-1 to migrate on rBM. Addition of recombinant stromelysin-1 to cells plated on 2D rBM substrata did not affect cell migration (Figure 7C). Moreover, cell migration on 2D rBM was unaffected by the inhibitor of matrix metalloproteinases GM6001 (Figure 7C), which abrogates rBM invasion by SCg6 cells (Lochter *et al.*, 1997b). Thus matrix metalloproteinases in general and stromelysin-1 in particular are not required for migration of SCg6 cells on rBM but, instead, modulate the invasive behavior of these cells by regulating proteolysis.

When we transfected SCg6 cells with stromelysin-1 promoter–luciferase reporter constructs, antibodies against α1 and α2, but not α6 or β1, integrins inhibited activity of the stromelysin-1 promoter in cells maintained on rBM, on tissue culture plastic, or in suspension culture (Figure 8, A, C, and D). Activity of the control RSV promoter construct, which was cotransfected with the stromelysin-1 promoter construct into the same cell population, was not inhibited by any of the antibodies (Figure 8B). Thus, the antibodies directed against α1 and α2 integrins interfered in the signaling pathway that regulates transcription of the stromelysin-1 gene.

Discussion

Most cells have a large repertoire of integrins, yet individual integrins may play distinct roles in regulating proliferation, differentiation, and apoptosis (Hynes, 1992; Giancotti, 1997). Integrins play a central role in tumor cell invasion (Dedhar, 1995; Juliano, 1996). Because integrins could modulate invasion by affecting cell adhesion, cell motility, and gene expression, the molecular mechanisms underlying integrin function can only be understood by dissecting the function of individual integrins. In this study, we showed that different β1 integrins have distinct functions in cell adhesion, motility, and MMP expression in an aggressive tumor cell model system and that they act in concert to modulate invasive activity.

Antibodies against the β1 integrin subunit that block the interaction of all β1-containing integrins with ECM ligands completely inhibited the ability of SCg6 mammary carcinoma cells to invade rBM (Matrigel). These antibodies also abrogated cell migration along two-dimensional rBM and laminin substrata, impaired cell spreading on rBM, and reduced cell adhesion to laminin and type IV collagen without affecting plating efficiency or viability on rBM. The rBM contains a heterogenous mixture of ECM molecules (Kleinman *et al.*, 1986) that could serve as ligands for integrin receptors that do not contain the integrin β1 subunit and for nonintegrin ECM receptors such as cell surface proteoglycans (Timpl, 1993; Mercurio, 1995). These receptors appear to be sufficient for maintaining adhesion and survival in the absence of functional β1 integrins.

Although antibodies against β1 integrins interfered with tumor cell invasion by inhibiting various aspects of cell–ECM interactions, antibodies against α6 integrin, a major laminin receptor (Hynes, 1992), affected cell migration and invasion but not adhesion or morphology of cells maintained on rBM substrata. Thus, α6 integrins appear to facilitate SCg6 tumor cell invasion selectively by promoting tumor cell migration. The predominant α6 integrin expressed by SCg6 cells was α6β1 and not α6β4, the major α6-containing integrin of nonmalignant mammary epithelial cells in culture (Delcommenne and Streuli, 1995; Lochter *et al.*, 1997a; Stahl and Mueller, 1997; Weaver *et al.*, 1997). Whereas loss of α6β4 and increased expression of α6β1 are positively correlated with tumor cell aggressiveness in prostate cancer and in mammary carcinoma cells (Cress *et al.*, 1995), α6β4 and not α6β1 promotes tumor cell invasion in colorectal carcinoma (Chao *et al.*, 1996; Rabinovitz and Mercurio, 1996). And in several glioma cell lines, antibodies against α6 increase tumor cell invasion (Paulus and Tonn, 1994). It was also shown that integrin α6β1 is required for the interaction of murine melanoma cells with laminin (Hangan *et al.*, 1997). In contrast to our study, both tumor cell adhesion to and cell migration on laminin could be inhibited with the anti-α6 antibody GoH3. However, another antibody binding in close proximity to the GoH3 epitope on α6 only interfered with cell migration but not with cell adhesion. Thus, the α6β1 integrin appears to have distinct but overlapping functions in different tumor cell types.

Our data show that α1 and α2 integrins contribute to rBM invasion. A role for α1 integrins in tumor cell invasion has not been reported previously, but α2 integrins have been shown to be both stimulatory and inhibitory for invasion of different cell types (Zutter *et al.*, 1995; Vihinen *et al.*, 1996). The mechanism by which α1 and α2 integrins affect tumor cell invasion in our study appears to be different from that of α6 and other β1-containing integrins. Antibodies against α2 integrins had little effect on plating efficiency or cellular morphology on rBM, on the ability of cells to migrate on two-dimensional rBM or laminin substrata, or on cell attachment to laminin or type IV collagen. Antibodies against α1 integrins inhibited SCg6 cell adhesion to type IV collagen by ~50% only, without affecting cell adhesion to laminin. Instead, α1 and α2 integrins selectively regulated expression of matrix metalloproteinase stromelysin-1 at the transcriptional level. The block in invasion by antibodies against α1 and α2 integrins could be overcome by addition of recombinant stromelysin-1, indicating that loss of stromelysin-1 was responsible for the loss-of-function. The amount of stromelysin-1 used to recover invasive activity was similar to the amount that the cells secreted in the

absence of anti-α1 or anti-α2 antibodies and similar to the amount secreted by functionally normal mouse mammary epithelial cells transfected with a stromelysin-1 cDNA (Lochter *et al.*, 1997a,b; our unpublished results), a manipulation that resulted in acquisition of premalignant properties in these cells (Lochter *et al.*, 1997a). Whereas the recombinant stromelysin-1 was activated before its use in cell culture, most of the stromelysin-1 produced by mouse mammary carcinoma cells is in its latent form. We have argued previously that stromelysin-1 is locally activated by cells at sites where basement membrane degradation is required for invasion (Lochter *et al.*, 1997b). This is supported by the fact that, when a latent form of recombinant stromelysin-1 was used in the invasion assay, inhibition of invasion by antibodies against α1 and α2 integrins could also be overcome, although less efficiently than with activated stromelysin-1 (our unpublished results). Our data additionally support our previous observations that stromelysin-1 plays a critical role in mouse mammary tumor cell progression (Lochter *et al.*, 1997a) and is indispensable for rBM invasion by mouse mammary carcinoma cells (Lochter *et al.*, 1997b).

The antibodies against α1 and α2 integrins used in this study are function blocking (Miyake *et al.*, 1994; Mendrick *et al.*, 1995; Noto *et al.*, 1995) rather than function stimulating. Thus, it is likely that these antibodies inhibit stromelysin-1 expression by interfering with integrin binding to a ligand produced endogenously. Laminin, which is expressed by SCg6 cells (Galosy, Werb, Bissell, unpublished results), is the most likely candidate to mediate regulation of stromelysin-1 expression by signaling via α1β1 and α2β1 integrins. Laminin positively modulates stromelysin-1 expression (Lochter *et al.*, 1997b), whereas type I collagen, type IV collagen, and tenascin- C, which are other potential ligands for α1β1 and α2β1 integrins, do not affect stromelysin-1 expression (Lochter *et al.*, 1997b; our unpublished results). Therefore, we propose that the basal level of stromelysin-1 expression in SCg6 cells is maintained by the binding of endogenously produced laminin to α1β1 and α2β1 integrins and that stromelysin-1 expression increases as a result of further receptor occupancy by exogenous laminin (or rBM) (Lochter *et al.*, 1997b). Both of these processes would then be blocked by antibodies against α1 and α2 integrins. Antibodies against β1 integrins, which should also block integrins α1β1 and α2β1, were without effect on stromelysin-1 protein expression or promoter activity. It is at present not clear why this is so; however, one possible explanation is that antibodies against β1 will block other integrins that affect processes that negatively impact on stromelysin-1 expression, leading to simultaneous inhibition of integrins that promote stromelysin-1 production (α1β1 and α2β1) and of others that reduce it and thus neutralizing the effect of individual integrins on stromelysin-1 expression. One such integrin may be α3β1 that is expressed by SCg6 cells (our unpublished results) and mediates cell adhesion to laminin in other cell types (Gehlsen *et al.*, 1989; Mercurio, 1995). Interestingly, in two glioblastoma cell lines, antibodies against α3β1 integrin stimulated expression of matrix metalloproteinase gelatinase A (Chintala *et al.*, 1996), and increased gelatinase A production was associated with increased invasiveness of these cells (Chintala *et al.*, 1996). However, because of the lack of blocking antibodies that react with the murine α3β1 integrin, we were unable to investigate the role of α3β1 in invasion and metalloproteinase production of SCg6 cells. Alternatively, the antibodies may have different effects on integrins, blocking in one case

but maintaining the integrin in an active conformation, whereas in the other case, the integrin may be locked in an inactive conformation that would signal differently.

The involvement of integrins in regulation of matrix metalloproteinase expression is well documented (Werb *et al.*, 1989; Seftor *et al.*, 1992; Larjava *et al.*, 1993; Huhtala *et al.*, 1995; Riikonen *et al.*, 1995; Kheradmand *et al.*, 1998). In a recent study, it was shown that antibodies against α2 integrins block collagenase-1 production that is required for keratinocyte migration on type I collagen substrata (Pilcher *et al.*, 1997). Our study provides the first direct demonstration that integrins modulate tumor cell invasion by altering expression of matrix metalloproteinase genes. These results provide a rationale for further investigation of how ECM molecules, ECM receptors, and ECM-degrading enzymes work together to bring about tumor progression.

Acknowledgments

We are grateful to R. Galardy and Glycomed Corporation for providing GM6001 and GM1210, to J.S. Mudgett (Merck Research Laboratories) for the gift of stromelysin-1 genomic DNA clones, to J. Muschler for critical reading of the manuscript, and to Debbie Lam and Lana Spivak for excellent technical assistance. This work was supported by funds from the United States Department of Energy Office of Health and Environmental Research (contracts DE-AC03-76-SF00098) and the National Cancer Institute (grant CA-57621) and by a postdoctoral fellowship from the California Breast Cancer Research Program to A.L.

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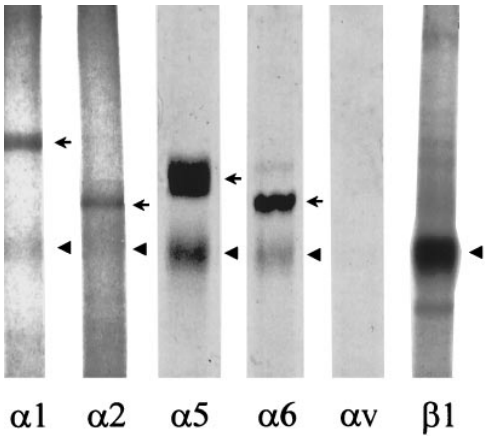
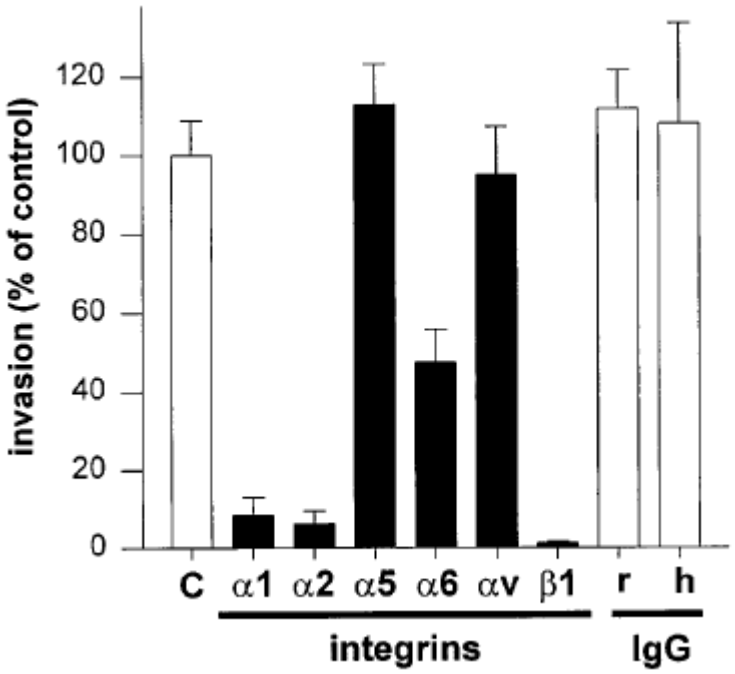
Figures

FIGURE 1



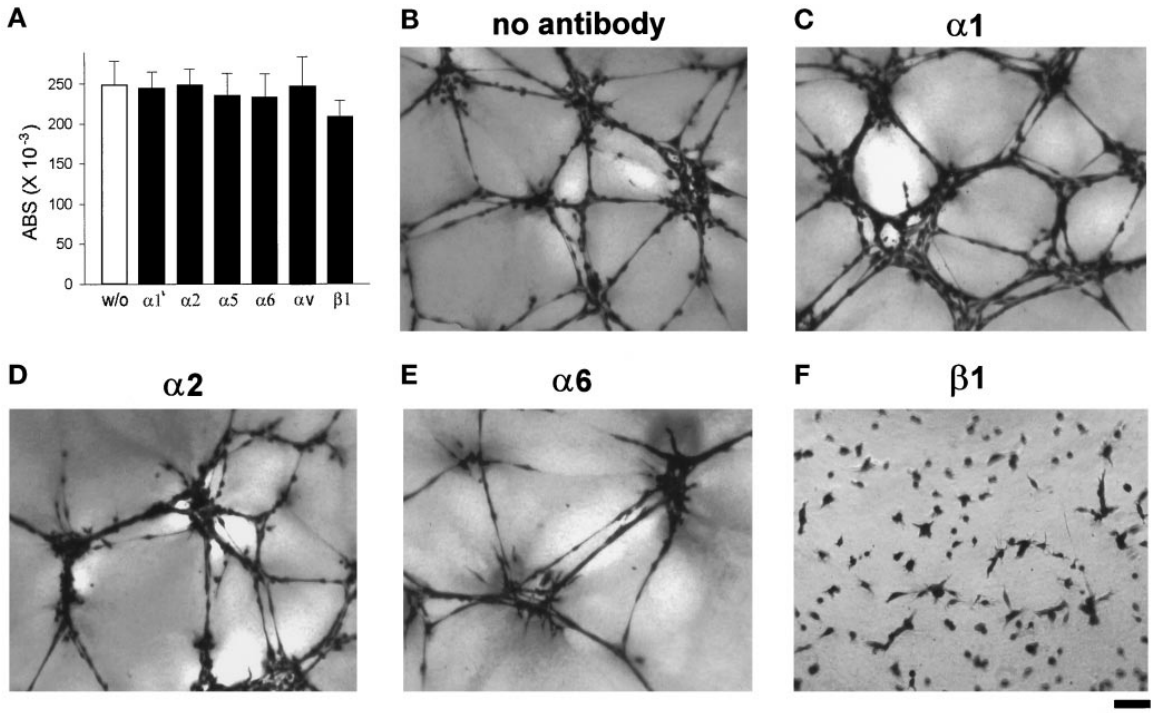
Integrin expression by SCg6 cells. Autoradiogram of nonreducing SDS-polyacrylamide gels run on immunoprecipitations performed with antibodies against α1, α2, α5, α6, αv, and β1 integrin subunits on radiolabeled cell lysates of SCg6 cells is shown. Note that, except for αv, all integrin subunits examined could be detected in SCg6 cells and that all antibodies coimmunoprecipitated a band corresponding to the expected molecular mass (130 kDa) of the β1 integrin subunit (◀). Positions (←) of integrin subunits α1 at 180 kDa, α2 at 150 kDa, α5 at 160 kDa, and α6 at 150 kDa are indicated.

FIGURE 2



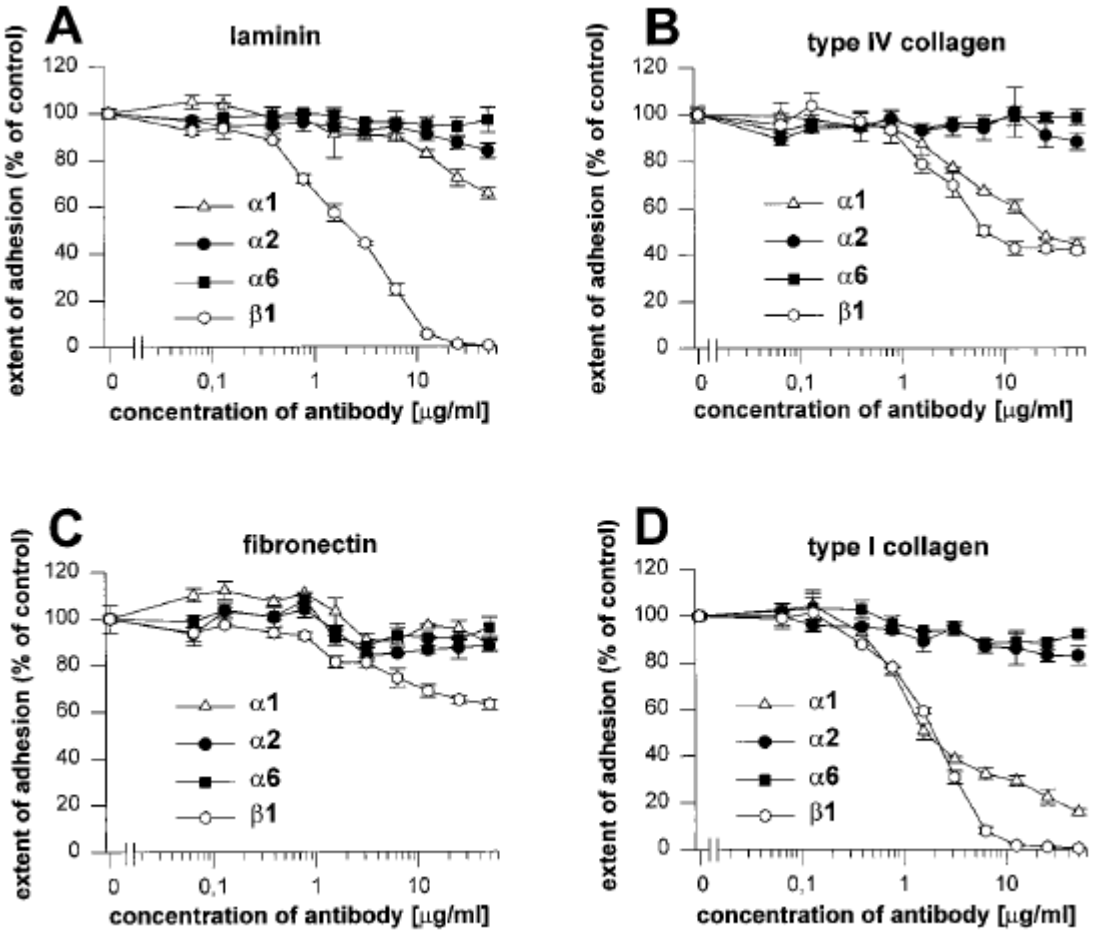
Effects of antibodies against α1, α2, α6, and β1 integrin subunits on the invasion of rBM by SCg6 cells. Invasion assays were performed for 24 h in modified Boyden chambers coated with rBM. SCg6 cells were then fixed and stained, and the number of cells that had migrated through rBM was counted by microscopic inspection. Invasion assays were performed in the absence of antibodies (control [C]), in the presence of antibodies against α1, α2, α5, α6, αv, or β1 integrin subunits, or in the presence of rat (r) or hamster (h) IgGs. Results are normalized with the number of cells migrating through rBM in the absence of antibodies set to 100. Means $\pm$ SD from three independent experiments are shown. In the absence of IgGs, 169.5 $\pm$ 8.89 cells/mm^2 migrated through rBM.

FIGURE 3



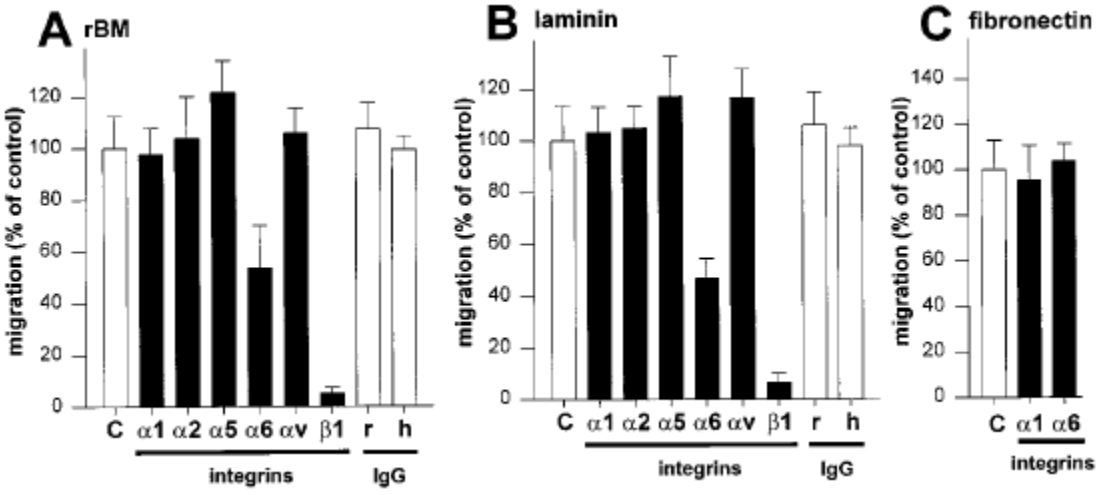
Cell number and morphologies of SCg6 cells maintained on rBM gels in the presence of antibodies against integrins. (A) SCg6 cells were maintained for 2 d on rBM, and the number of viable cells was determined by the MTT method. Means ± SD from three independent experiments are expressed as the absorbance (ABS) measured at 570 nm. Note that only minor differences in the number of viable cells were observed for the different antibody treatments. w/o, without antibody. (B–F) Micrographs of toluidine blue-stained SCg6 cells maintained for 2 d on rBMin the absence of antibodies (B) or in the presence of antibodies against α1 (C), α2 (D), α6 (E), and β1 (F) integrins are shown. Bar, 100 μm.

FIGURE 4



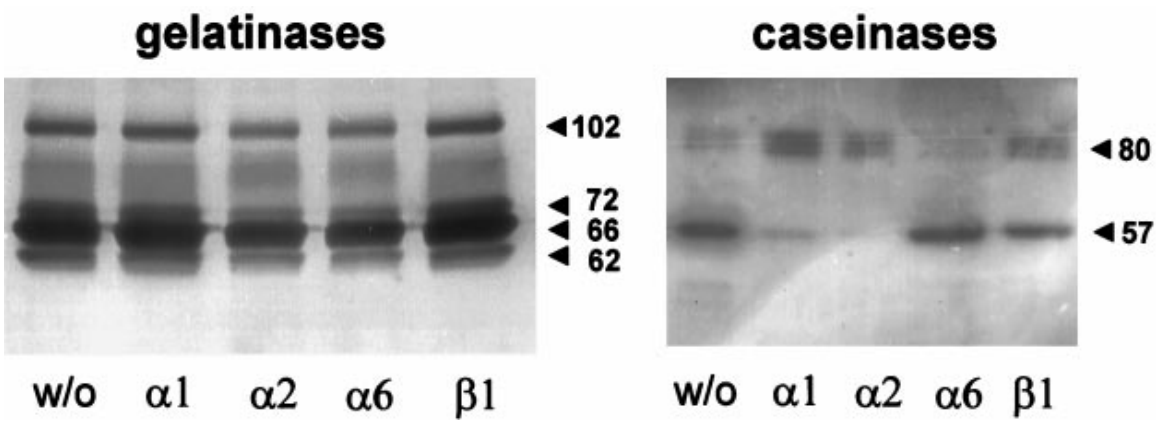
Effect of integrin antibodies on adhesion of SCg6 cells to ECM constituents. Adhesion assays on substrata coated with laminin (A), type IV collagen (B), fibronectin (C), or type I collagen (D) were performed for 1 h in the presence of increasing concentrations of antibodies against α1 (Δ), α2 (●), α6 (■), or β1 (○) integrin subunits. Results are normalized with the number of cells adhering to ECM substrata in the absence of antibodies set to 100. Means ± SD from three independent experiments are shown.

FIGURE 5



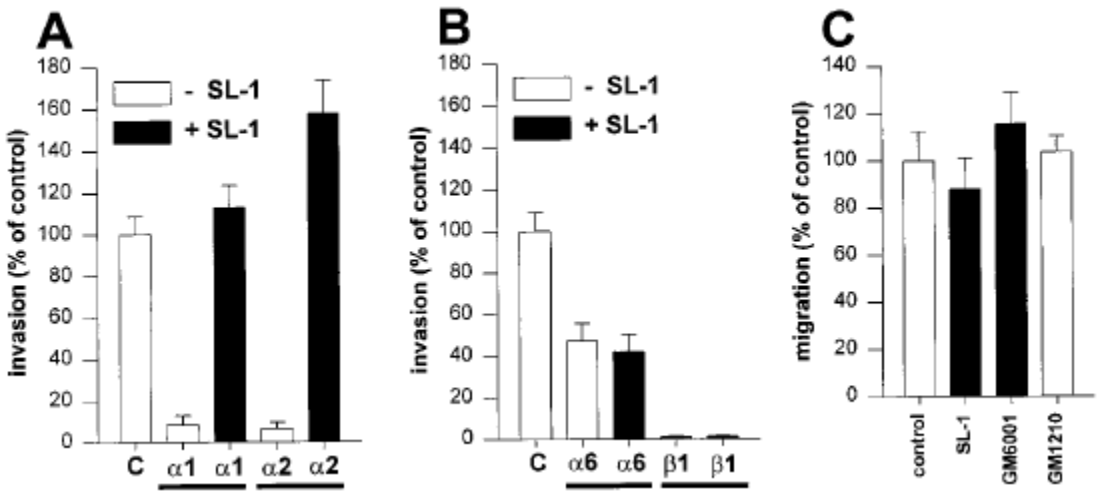
Effects of antibodies against α6 and β1 integrins on SCg6 cell migration along 2D rBM and laminin substrata. Cell migration assays were performed for 6 h in modified Boyden chambers coated with rBM (A), laminin (B), or fibronectin (C). SCg6 cells were then fixed and stained, and the number of cells that had migrated along the ECM was counted by microscopic inspection. Cell migration assays were performed in the absence of antibodies (control [C]), in the presence of antibodies against α1, α2, α5, α6, αv, or β1 integrin subunits, or in the presence of rat (r) or hamster (h) IgGs. Results are normalized with the number of cells that had migrated along the ECM in the absence of antibodies set to 100. Means $\pm$ SD from three independent experiments are shown. In the absence of IgGs, the number of cells migrating per square millimeter was 60.1 $\pm$ 7.55 on 2D rBM, 90.3 $\pm$ 12.25 on laminin, and 102.9 $\pm$ 13.27 on fibronectin.

FIGURE 6



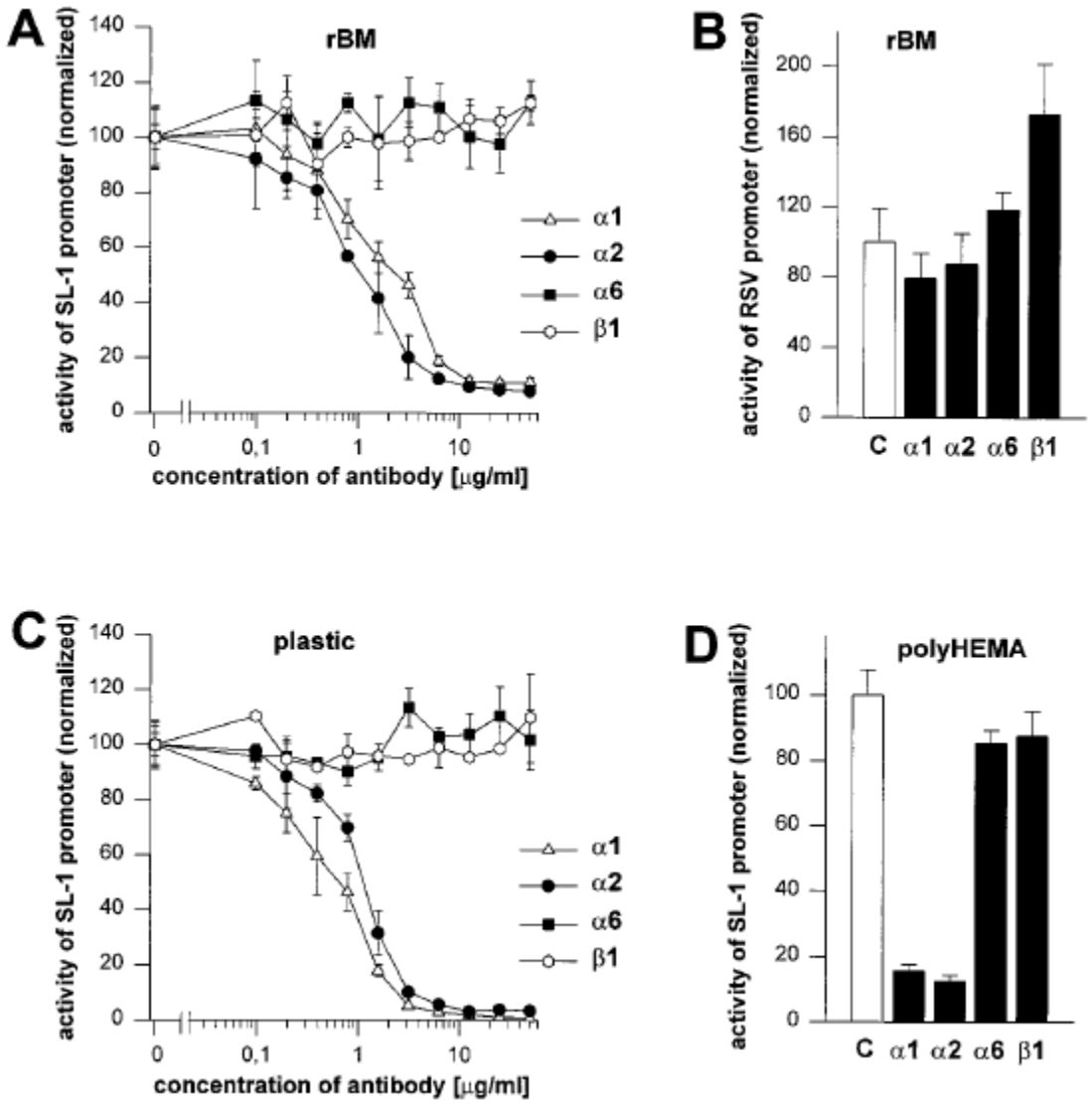
Effect of antibodies against integrins on the production of ECM-degrading proteinases. Medium conditioned by SCg6 cells maintained for 2 d on rBMin the absence of antibodies (w/o) or in the presence of antibodies against α1, α2, α6, or β1 integrin subunits was analyzed by gelatin and casein substrate gel zymography. Negative images of zymograms are shown. Molecular masses of major gelatinolytic and caseinolytic bands are indicated. Left, gelatinases of 72, 66, and 62 kDa represent gelatinase A, and the 102-kDa gelatinase represents gelatinase B. Right, the 57-kDa caseinase represents stromelysin-1, and the 80-kDa caseinase represents an unidentified metalloproteinase secreted by SCg6 cells (Lochter *et al.*, 1997b).

FIGURE 7



Effect of recombinant stromelysin-1 (SL-1) on invasion and migration of SCg6 cells. (A and B) Invasion assays were performed for 24 h in modified Boyden chambers coated with rBM gels. SCg6 cells were then fixed and stained, and the number of cells that had migrated through rBM was counted by microscopic inspection. Invasion assays were performed in the absence of antibodies (control [C]) or in the presence of antibodies against α1, α2, α6, or β1 integrin subunits with (+ SL-1, black bars) or without (- SL-1, white bars) the addition of recombinant stromelysin-1 to the culture medium. Results are normalized with the number of cells migrating through rBM in the absence of antibodies and stromelysin-1 set to 100. Means $\pm$ SD from three independent experiments are shown. In the absence of antibodies, 183.2 $\pm$ 16.21 cells/mm^2 migrated through rBM. (C) Two-dimensional cell migration assays were performed for 6 h in modified Boyden chambers coated with rBM. SCg6 cells were then fixed and stained, and the number of cells that had migrated along rBM was counted by microscopic inspection. Cell migration assays were performed in the absence of additives (control) or in the presence of recombinant stromelysin-1, the matrix metalloproteinase inhibitor GM6001, or its inactive homologue GM1210. Results are normalized with the number of cells that had migrated along rBM in the absence of additives set to 100. Means $\pm$ SD from three independent experiments are shown. Under control conditions, 72.8 $\pm$ 9.16 cells/mm^2 migrated along rBM.

FIGURE 8



Effect of antibodies against α1 and α2 integrins on stromelysin-1 promoter activity in SCg6 cells. Luciferase (A, C, and D) and β-galactosidase (B) activity of SCg6 cells cotransfected with SL-1 promoter–luciferase and RSV promoter–β-galactosidase constructs was analyzed 2 d after maintenance of cells on rBM gels (A and B), on tissue culture plastic (C), or in suspension culture (poly-HEMA, D). Cells were cultured in the presence of increasing concentrations of antibodies against α1 (Δ), α2 (●), α6 (■), or β1 (○) integrin subunits (A and C) or in the presence of 10 μg/ml antibodies (B and D). Results are normalized with values obtained for cells maintained in the absence of antibodies (control [C] in B and D) set to 100. Means ± SD from three independent experiments are shown. In the absence of antibodies, the average number of relative light units obtained per well was 22371 on rBM, 6580 on plastic, and 20689 on poly-HEMA for the stromelysin-1 promoter and 9618 on rBM for the RSV promoter.