A Role for the G₁₂ Family of Heterotrimeric G Proteins in Prostate Cancer Invasion

Many studies have suggested a role for the members of the G₁₂ family of heterotrimeric G proteins (G₁₂₀ and G₁₂₃) in oncogenesis and tumor cell growth. However, few studies have examined G₁₂ signaling in actual human cancers. In this study, we examined the role of G₁₂ signaling in prostate cancer. We found that expression of the G₁₂ proteins is significantly elevated in prostate cancer. Interestingly, expression of the activated forms of G₁₂₀ or G₁₂₃ in the PC3 and DU145 prostate cancer cell lines did not promote cancer cell growth. Instead, expression of the activated forms of G₁₂₀ or G₁₂₃ in these cell lines induced cell invasion through the activation of the RhoA family of G proteins. Furthermore, inhibition of G₁₂ signaling by expression of the RGS domain of the p115-Rho-specific guanine nucleotide exchange factor (p115-RGS) in the PC3 and DU145 cell lines did not reduce cancer cell growth. However, inhibition of G₁₂ signaling with p115-RGS in these cell lines blocked thrombin- and thromboxane A₂-stimulated cell invasion. These observations identify the G₁₂ family proteins as important regulators of prostate cancer invasion and suggest that these proteins may be targeted to limit invasion- and metastasis-induced prostate cancer patient mortality.

It is estimated that over 230,000 new cases of prostate cancer will be diagnosed in the United States this year (1). Although the prognosis for patients with early stage prostate cancer has improved, the treatment options for patients with locally advanced disease or metastasis remain few. For this reason, prostate cancer remains the second leading cause of cancer deaths in males and will claim the lives of greater than 25,000 American men this year alone (1). Therefore, it is imperative that new strategies be developed to treat patients with advanced prostate cancer, and this requires a better understanding of the molecular mechanisms that regulate prostate cancer invasion and metastasis.

Studies over the past three decades have clearly established that signaling through cell surface G protein-coupled receptors (GPCRs) controls many physiologic and pathophysiologic processes (2, 3). However, it is only recently that the functional significance of GPCRs in prostate cancer invasion and metastasis has begun to be appreciated (4). GPCRs for thrombin (5), thromboxane A₂ (6, 7), bradykinin (8), lysophosphatidic acid (9), and SDF-1 (10) have all been implicated in prostate cancer invasion and metastasis. The best characterized example is the thrombin receptor, protease-activated receptor-1 (PAR-1). PAR-1 is preferentially expressed in aggressive prostate cancer lines and in metastatic prostate cancer specimens (5, 11, 12). Moreover, studies suggest that the activation of PAR-1 increases prostate cancer cell resistance to apoptotic stimuli (5), stimulates the expression of angiogenic factors (13), and promotes prostate cancer cell invasion (14, 15). Nevertheless, the pathways through which PAR-1 and the GPCRs mentioned above affect prostate cancer cell function are not fully understood.

GPCRs alter cellular function primarily through the activation of heterotrimeric G proteins. Heterotrimeric G proteins consist of two functional signaling units, a guanine nucleotide binding α-subunit and a βγ-subunit dimer. The α-subunits of heterotrimeric G proteins can be divided into four families based on sequence homology: Gₛ, G₁₁, G₁₂, and G₁₃ (4, 16, 17). The last of the four families to be identified, the G₁₃ family has been of particular interest to cancer researchers, since its members were found to promote the growth and oncogenic transformation of murine fibroblasts (18, 19). These findings led to the hypothesis that GPCRs may signal through the G₁₃ proteins to promote tumorigenesis and tumor cell growth (20). Interestingly, however, studies that examined the role of the G₁₃ proteins in development found that G₁₃ proteins were not required for cell growth but were critical for cell movement in the developing embryo (21–25). Since similar cellular movements underlie cancer cell invasion (26–29), these findings suggest that G₁₃ signaling may also play a role in cancer metastasis.

Recently, we examined the role of the G₁₃ proteins in human breast cancer. We found that the G₁₃ proteins promote breast cancer metastasis by stimulating cancer cell invasion, not cancer cell growth (30). In this study, we investigated the role of G₁₃ signaling in prostate cancer. We found that the G₁₃ proteins are
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up-regulated in prostate cancer and that signaling through the G12 pathway does not increase prostate cancer cell growth. Rather, activation of G12 induces a striking increase in cancer cell invasiveness. These observations identify G12 family proteins as regulators of prostate cancer invasion and provide support for targeting these proteins in therapeutic strategies to limit invasion- and metastasis-induced patient morbidity and mortality.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Antibodies to RhoA, Gaq, Gab12, and Gab13, and the blocking peptide for the Gab12 antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-Myc antibody was obtained from Zymed Laboratories (San Francisco, CA). Polyclonal antisera to Gab12 and Gab13 were also obtained from Dr. Stefan Offermanns (University of Heidelberg). Polyclonal antiserum to RGS2 was from Dr. David Siderovski (University of North Carolina, Chapel Hill, NC). Polyclonal antiserum to Gab1 was from Dr. Tom Gettys (Pennington Biomedical Research Center, Baton Rouge, LA). Recombinant human thrombin was from Enzyme Research Laboratories (South Bend, IN), and U46619 and tetanoylin were from Biomol (Plymouth Meeting, PA). Growth factor-reduced Matrigel was from BD Biosciences, and the fibronectin, from human plasma, was from Sigma.

Cell Lines—The PC3, DU145, and LNCaP cell lines were obtained from the Duke University Medical Center Cell Culture Facility. The immortalized prostate epithelial cells (PrEC) (31) were obtained from Dr. Phillip Febbo (Duke University, Durham, NC). The PC3 cell line was maintained in F-12K nutrient mixture (Invitrogen) supplemented with 10% fetal bovine serum. The LNCaP and DU145 cell lines were maintained in RPMI (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum. The PrEC-LHS cells were maintained in a defined medium (PrEGM) from BioWhittaker (Rockland, ME).

Adenoviral Infections—The dominant negative Rho kinase adenovirus was obtained from Dr. P. Vasantha Rao (Duke University, Durham, NC). The other recombinant adenoviruses were constructed by subcloning human Gaq (Q209L), Gab12 (Q231L), Gab13 (Q226L), and HA-RGS2, all from the UMR cDNA Resource (Rolla, MO), Myc-p115-RGS (gift of Dr. Tohru Kosaza, University of Illinois, Chicago, IL), and Myc-p115RGS (E29K) (generated by site-directed mutagenesis of the Myc-p115) into the Adtrack-CMV vector (gift of Dr. Bert Vogelstein, Johns Hopkins University Medical Center, Baltimore, MD) and then recombinating these with pAdEasy-1 in the BJ5183 strain of Escherichia coli (Strategene, La Jolla, CA). The resulting DNA was transfected into HEK 293 cells with Lipofectamine (Invitrogen), and the viruses were serially amplified and purified using Adeno-X™ virus purification kits (BD Biosciences). Cell lines were infected at a multiplicity of 26484 units of thrombin (Enzyme Research Laboratories, South Bend, IN) in 50 mM Tris-HCl, pH 7.7, 14 mM β-mercaptoethanol, 150 g/ml Geneticin (Invitrogen).

Cell Invasion Assay—For invasion assays, transwell chamber filters (8-μm pore size, polycarbonate filter, 6.5-mm diameter; Costar) were coated with 50 μg of growth factor-reduced (GFR) Matrigel™. After infection with adenovirus, cells were starved for 12 h in Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin, detached with Cellstripper™ (Mediatech, Herndon, VA), and 5 × 10⁵ cells in 100 μl were placed into the upper chamber of the transwell with or without agonists. For experiments using C3 toxin, 1 mg of purified C3, and 20 hemolytic units of tetanoylin were added to the cells for 1 h prior to harvesting the cells. For experiments with thrombin or U46619, the cells were treated at the indicated ligand concentration for 2 h prior to harvest and for the duration of the experiment. The upper well of the transwell was then transferred to a well containing 600 μl of 5 μg/ml of fibronectin diluted in Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin. Cells were incubated for 36 h at 37 °C in a humidified incubator. Cells in the top well were removed with cotton swabs. The membranes were then stained (Hema3 staining kit; Fisher), and the cells were counted using a phase-contrast microscope. Five randomly selected high powered fields were counted for each membrane.

Preparation of GST Fusion Proteins—GST-C3 expression construct was obtained from Judith Meinkoth (University of Pennsylvania, Philadelphia, PA), and the GST-rhoetkin-RBD construct was obtained from Robert Lefkowitz (Duke University Medical Center, Durham, NC). The GST-C3 and GST-rhoetkin-RBD proteins were made in the BL21DE3 strain of E. coli (Invitrogen). Briefly, starter cultures from a transformed bacterial colony were grown for 16 h and then used to inoculate 500 ml of LB and grown at 37 °C for 2–3 h until the optical density reached 0.5–0.6. At this point, the cells were induced with 0.5 mM isopropyl-d-thiogalactopyranoside (Sigma), and cultures were grown for an additional 2.5 h at 37 °C. The cells were harvested by centrifugation for 15 min at 6,000 × g at 4 °C, and the resulting pellet was resuspended in 2.5 ml of buffer A (2.3 mM sucrose, 50 mM Tris-HCl, pH 7.7, 1 mM EDTA, and Complete Mini, EDTA-free protease inhibitor mixture tablets (Roche Applied Science)) followed by dilution with 10 ml of buffer B (50 mM Tris-HCl, pH 7.7, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and a 1:500 protease inhibitor mix). The cells were then passed three times at 10,000 p.s.i. through a microfluidizer (Microfluidics Corp., Newton, MA). The lysates were cleared by centrifugation at 30,000 × g for 30 min, and the resulting supernatant was incubated with glutathione-Sepharose 4B beads (Amersham Biosciences) equilibrated in buffer B for 2 h at 4 °C with continuous rocking. Finally, the beads were washed three times in Buffer B. The C3 toxin was cleaved from the GST domain by gently rocking the beads overnight at 4 °C with 10 units of thrombin (Enzyme Research Laboratories, South Bend, IN) in 50 mM Tris-HCl, pH 7.7, 14 mM β-mercaptoethanol, 150 g/ml Geneticin. The GST-C3 construct was cleaved from the GST domain by gently rocking the beads overnight at 4 °C with 10 units of thrombin (Enzyme Research Laboratories, South Bend, IN) in 50 mM Tris-HCl, pH 7.7, 14 mM β-mercaptoethanol, 150 g/ml Geneticin.
mm NaCl, and 2.5 mm CaCl$_2$. The cleaved product was then dialyzed into phosphate-buffered saline, visualized by SDS-PAGE with Coomassie Blue staining, and stored in aliquots at −80°C. Protein concentration was determined by Bradford assay (Bio-Rad).

**Immunohistochemistry**—Institutional Review Board-approved prostate samples were from Ardaics Co. (Lexington, MA). The tissue microarray (catalog no. BR801) was from US Biomax (Rockville, MD). Following paraffin removal and quenching of endogenous peroxidase, 5-μm sections were steamed in 10 mm citrate, pH 6.0, for 15 min in a steamer (catalog no. HS900; Black & Decker) and then incubated with Background Buster® (Innovex Biosci, Richmond, CA) for 30 min. Sections were then incubated with $\alpha_{12}$ antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100 in phosphate-buffered saline for 1 h, followed by biotinylated goat anti-rabbit antisera (Vector Laboratories, Burlingame, CA) diluted 1:200 in phosphate-buffered saline for 30 min, followed by horseradish peroxidase-labeled streptavidin (Jackson ImmunoResearch, West Grove Park, PA) for 30 min, all at room temperature. Bound immune complex was visualized with diaminobenzidine (Innovex Biosci, Richmond, CA); hematoxylin counterstain (Fisher) was used. The $\alpha_{12}$ staining was graded 0–3+ based on intensity by two independent board-certified pathologists (T. A. F. and J. F. M.), and data were analyzed using one-way analysis of variance and Dunn’s multiple comparison test in Prism version 4.0c (GraphPad, San Diego, CA).

**RESULTS**

$\alpha_{12}$ Proteins Are Up-regulated in Invasive, Tumorigenic Prostate Cancer Cell Lines—To assess the biologic significance of the $\alpha_{12}$ family of heterotrimeric G proteins in prostate cancer, we compared expression of $\alpha_{12}$ and $\alpha_{13}$ in an immortalized prostate epithelial cell line (PrEC LHS) (31) and in the three commonly used prostate cancer cell lines: LNCaP, DU145, and PC3. Interestingly, $\alpha_{12}$ and $\alpha_{13}$ expression was significantly higher in the more tumorigenic and invasive DU145 and PC3 (32) cell lines than in the less tumorigenic, noninvasive LNCaP cell line (33) or in the nontransformed prostate epithelial cell line (Fig. 1). This up-regulation appeared to be particularly pronounced for the $\alpha_{12}$ protein (Fig. 1). These data provided the initial evidence that increased $\alpha_{12}$ signaling may be associated with increased tumorigenicity and/or invasiveness of these two prostate cancer cell lines.

$\alpha_{12}$ Is Up-regulated in Pathologic Specimens of Adenocarcinoma of the Prostate—To determine whether the *in vitro* findings that $\alpha_{12}$ expression is elevated in aggressive prostate cancer cells extended to actual human tissues, we performed immunohistochemical analysis of $\alpha_{12}$ expression in histopathologic specimens taken from patients with adenocarcinoma of the prostate. Anti-$\alpha_{12}$-stained sections of prostate revealed that prostate cancer cells consistently expressed higher levels of $\alpha_{12}$ protein compared with benign prostate epithelial cells within the same tissue section (Fig. 2). $\alpha_{12}$ staining could be completely blocked by preincubation of the antibody with its blocking peptide, demonstrating antibody specificity (supplemental Fig. 1). Further, staining of these same sections with an anti-$\alpha_{13}$ antibody demonstrated that benign prostate epithelial cells and prostate cancer cells express similar levels of $\alpha_{13}$ (data not shown), suggesting that this elevation in expression is specific to $\alpha_{12}$.
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### TABLE 1

**Distribution of prostate surgical specimen field characteristics by G\textsubscript{12} staining**

Sections from prostatectomy specimens (n = 13) were stained for G\textsubscript{12} as described under “Experimental Procedures.” Areas of benign epithelium, PIN, and carcinoma in sequential (×20) microscopic fields were scored 0–3+ based on staining intensity. The number of microscopic fields containing each of the diagnoses and their respective mean scores are indicated below.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Staining intensity</th>
<th>Average ± S.E.</th>
</tr>
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<tbody>
<tr>
<td>Benign prostate epithelium</td>
<td>0 126 79 31 7</td>
<td>0.67 ± 0.08</td>
</tr>
<tr>
<td>HG-PIN</td>
<td>126 4 22 46 53</td>
<td>2.19 ± 0.83*</td>
</tr>
<tr>
<td>Invasive CA</td>
<td>236 3 28 78 126</td>
<td>2.39 ± 0.74*</td>
</tr>
</tbody>
</table>

* p < 0.0001 for HG-PIN versus benign prostate epithelium.
* p < 0.0001 for invasive cancer versus benign prostate epithelium.

To broaden our analysis of G\textsubscript{12} expression in prostate cancer, a tissue microarray of 16 examples of normal prostate and 73 examples of invasive carcinoma with matched benign tissue was examined by immunohistochemistry, and the results were graded 0–3+ based on staining intensity. This analysis (not shown) demonstrated that G\textsubscript{12} expression is significantly increased in invasive carcinoma of the prostate; the staining intensity of normal prostate epithelium was 0.2 ± 0.1; the staining intensity for invasive prostate cancer was 1.6 ± 0.1. Since prostate intraepithelial neoplasms (PINs) were not adequately represented in the commercial tissue microarrays, we also performed the G\textsubscript{12} staining on 13 samples from radical prostatectomy cancer specimens obtained from our institution (Table 1). Staining of these sections confirmed that G\textsubscript{12} expression is increased in PIN as well as invasive carcinoma of the prostate; in this analysis, the staining intensity of normal prostate epithelium was 0.67 ± 0.05, the staining intensity of PIN was 2.2 ± 0.08, and the staining intensity of invasive prostate cancer was 2.4 ± 0.05. Together, these data support the conclusion that G\textsubscript{12} expression increases soon after neoplastic transformation of the prostate and before the tumors become invasive.

**G\textsubscript{12} Signaling Does Not Promote Prostate Cancer Cell Growth or Tumorigenesis**—Previous studies have suggested that G\textsubscript{12} signaling is able to promote fibroblast growth and tumorigenesis (18, 19, 34, 35). However, in breast cancer cells, G\textsubscript{12} signaling did not appear to promote cell growth or tumorigenesis (30). In order to determine the biologic significance of the G\textsubscript{12} proteins in prostate cancer, we first examined the effects of modulating the G\textsubscript{12} pathway on prostate cancer cell growth and tumorigenesis. In order to drive G\textsubscript{12} signaling in the DU145 and PC3 cell lines, we used adenovirus to express the activated forms of G\textsubscript{12} (G\textsubscript{12}Q226L) and G\textsubscript{13} (G\textsubscript{13}Q226L). Expression of these activated variants had no effect on in vitro prostate cancer cell growth (supplemental Fig. 2, A and B). In addition, in order to inhibit G\textsubscript{12} signaling, we used adenovirus to express the RGS domain of the p115-Rho-specific guanine nucleotide exchange factor (RhoGEF) (p115-RGS). This domain selectively binds G\textsubscript{13} and G\textsubscript{13}, preventing them from interacting with their downstream effectors (36, 37). Expression of this inhibitor of G\textsubscript{12} signaling also had no effect on prostate cancer cell growth (supplemental Fig. 2, A and B). To examine the effects of G\textsubscript{12} signaling on prostate cancer cell tumorigenesis, recombinant retroviruses were used to stably express either G\textsubscript{12} (Q231L) or the p115-RGS in the PC3 cell line. Interestingly, expression of neither of these proteins had any effect on the growth of PC3 cells in soft agar (supplemental Fig. 2C) or when implanted into the flanks of immunocompromised mice (data not shown). Taken together, these results suggest that G\textsubscript{12} signaling does not affect prostate cancer cell growth or tumorigenesis.

**G\textsubscript{12} Signaling Promotes Prostate Cancer Cell Invasion**—Since previous studies have suggested a role for the G\textsubscript{12} proteins in cell migration (21–25) and cancer cell invasion (30), we next examined the role of G\textsubscript{12} signaling in prostate cancer cell invasion. We found that expression of the activated forms of G\textsubscript{12} or G\textsubscript{13} in the PC3 (Fig. 3A) and DU145 (Fig. 3B) cell lines significantly increased the ability of these cells to invade a Matrigel\textsuperscript{TM} barrier reconstituted in a transwell migration chamber. Since most receptors that couple to the G12 family of proteins are members of the RhoA family of monomeric GTPases, the G\textsubscript{12} proteins stimulate Rho activity principally through the direct interaction with a family of RhoGEFs that includes p115-RhoGEF (38), PDZ-RhoGEF (39), and LARG (40). G\textsubscript{12} and G\textsubscript{13} fail to promote cellular invasion in either prostate cancer cell line (Fig. 3, A and B), suggesting that this ability to promote prostate cancer invasion is specific to the G\textsubscript{13} family.

**G\textsubscript{12} Signaling Promotes Prostate Cancer Cell Invasion through a Rho-dependent Pathway**—The best characterized downstream effectors of the G\textsubscript{12} family of heterotrimeric G proteins are members of the RhoA family of monomeric GTPases. The G\textsubscript{12} proteins stimulate Rho activity principally through the direct interaction with a family of RhoGEFs that includes p115-RhoGEF (38), PDZ-RhoGEF (39), and LARG (40). G\textsubscript{12} and G\textsubscript{13} bind to these RhoGEFs through an N-terminal RGS motif, recruiting them to the membrane, where they are able to promote Rho activation (38–40). Since many studies have demonstrated that the Rho family of proteins and their downstream effectors play a significant role in prostate cancer invasion (41), we examined the role of Rho signaling in G\textsubscript{12}-induced prostate cancer invasion.

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**FIGURE 3. Expression of the activated form of G\textsubscript{12} (G\textsubscript{12}Q226L) or G\textsubscript{13} (G\textsubscript{13}Q226L) but not G\textsubscript{13}Q226L induces prostate cancer cell invasion in vitro.** PC3 (A) and DU145 (B) cells were transduced with the indicated adenovirus, starved for 18 h, and then allowed to invade GFR Matrigel-coated transwell filters for 30 h. A and B, immunoblot analysis shows expression levels of G\textsubscript{12}, G\textsubscript{12}Q226L, and G\textsubscript{13} in the PC3 cell line after infection with the indicated adenovirus as an example of protein expression. α-tubulin was used as a loading control. A and B, graph shows fold-increase in invasion over GFP control. Experiments were performed in duplicate, and at least four fields were counted for each replicate. All experiments were performed at least three times. All results are presented as mean ± S.E. *p < 0.05 as determined by paired Student’s t test. IB, immunoblot.
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First, we confirmed that $G_{12}$ signaling is able to activate Rho in prostate cancer cells. Expression of the activated forms of $G_{12}$ or $G_{13}$ induced a significant increase in the levels of GTP-bound RhoA in both the PC3 (Fig. 4A) and DU145 cells (8). Cells were transduced with the indicated adenovirus and then starved for 18 h. Cells were lysed, and lysates were subjected to pull-down assays using a GST fusion of the activated RhoA-binding domain of rhoetekin. Levels of precipitated RhoA were determined by immunoblot analysis (IB) using anti-RhoA antibody. Levels of total RhoA, $G_{12}$, $G_{13}$, and $G_{13}$, were also determined as a control. All panels are representative of two or more separate experiments. Expression of $G_{12}$ in PC3 (C) and DU145 cell (D) invasion in through a Rho-dependent pathway. Cells were transduced with the indicated adenovirus, starved for 18 h, and then allowed to invade GFR Matrigel-coated transwell filters for 30 h. For experiments with C3 toxin, cells were treated with the recombinant toxin for 1 h prior to harvest. For experiments with Y29632, inhibitor (50 μM) was added at the time of plating to both the upper and lower wells of the transwell apparatus. Experiments were performed in duplicate, and results are presented as fold increase over that observed with GFP control. All experiments were performed at least three times. All results are presented as mean ± S.E. *p < 0.01; **p < 0.005 as determined by paired Student’s t test.

As expected, expression of the activated form of $G_{12}$ in the C3 toxin-positive DU145 cell line did not cause an increase in the level of GTP RhoA, whereas expression of the activated forms of $G_{12}$ or $G_{13}$ induced a significant increase in the level of GTP RhoA, and the expression of the activated form of $G_{12}$ induced a minor increase in the level of GTP RhoA (Fig. 5A).

To confirm that $G_{12}$ was able to inactivate E-cadherin in a Rho-independent fashion, activated $G_{12}$ and activated $G_{12}$ were expressed in the DU145 cells, and the cadherin function was analyzed using a so-called “fast aggregation” assay (42, 44). In this assay, the cells were trypsinized in the presence of 10 mM Ca$^{2+}$ to maintain cadherin integrity and then disassociated and allowed to aggregate for 1 h in the presence of 1 mM Ca$^{2+}$. Aggregate size and number were then scored as a measure of cadherin function. Inclusion of the Ca$^{2+}$ chelator EGTA in the assay completely disrupted aggregate formation (Fig. 5B), confirming that the cell-cell interactions observed were mediated by cadherins. Moreover, consistent with our previous findings (42), expression of either $G_{12}$ or $G_{12}$ significantly decreased the ability of the DU145 cells to aggregate (Fig. 5B), suggesting that $G_{12}$ signaling is able to inactivate E-cadherin in this cell line. Finally, we examined whether $G_{12}$-induced inactivation of E-cadherin was able to promote DU145 cell invasion independent of Rho activation. The activated forms $G_{12}$ and $G_{12}$ were expressed in the DU145 cells, and cellular invasion was assayed. Although both activated $G_{12}$ and $G_{12}$ (244–249) were able to disrupt E-cadherin-mediated adhesion, only activated $G_{12}$ induced prostate cancer cell invasion. Thus, although the $G_{12}$ to cadherin pathway appears to be intact in the DU145 cells, $G_{12}$-induced inactivation of E-cadherin does appear to be sufficient to stimulate invasion of this cell type.

$G_{12}$ Signaling Is Required for Thrombin- and Thromboxane A2-stimulated Invasion of Prostate Cancer Cells—As noted in the Introduction, studies have demonstrated that signaling through GPCRs for factors such as thrombin (15), thromboxane A2 (6), bradykinin (8), and lysophosphatidic acid (9) promotes prostate cancer cell invasion and metastasis. Interestingly, many of these GPCRs are known to couple to the $G_{12}$ family of heterotrimeric G proteins (45). Thus, we decided to determine whether $G_{12}$ signaling via Rho is involved in the invasion-promoting activity of these GPCRs. First, we confirmed that GPCR signaling does indeed elicit Rho activation in pros-
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A.

B.  

C.

FIGURE 5. G12-induced inactivation of E-cadherin is not sufficient to promote prostate cancer cell invasion. A, expression of the activated form of Gα12QL(Δ244–249) does not induce Rho activation in the DU145 cell line. DU145 cells were transduced with the indicated adenovirus and then starved for 18 h. Cells were lysed, and lysates were subjected to pull-down assays using a GST fusion of the activated RhoA-binding domain of rhotekin. Levels of precipitated RhoA were determined by immunoblot analysis (IB) using anti-RhoA antibody. Levels of total RhoA, Gα12, Gα13, and Gαq were also determined as a control. B, expression of Gα12, Gα13, and Gαq were also determined as a control. C, expression of the activated form of Gα12QL(Δ244–249) inhibits calcium-dependent aggregation of DU145 cells. DU145 cells were transduced with the indicated adenovirus, starved for 18 h, and then subjected to a 1-h “fast aggregation” assay (see “Experimental Procedures” for details). The percentage of cell aggregation was determined by counting the number of cells in each field in aggregates larger than 10 cells and dividing this by the total number of cells in each field. Data shown are representative of results obtained in three separate experiments. E-cadherin levels in the PC3 and DU145 cell lines were measured by immunohistochemistry. Immunohistochemical staining of benign prostate and prostate cancer specimens revealed that the expression of Gα12 is elevated in both prostate intraepithelial neoplasia and invasive prostate cancer, when compared with benign prostate epithelial cells. Taken together with the findings that the expression levels of both Gα12 and Gα13 are higher in aggressive prostate cancer cell lines compared with nontransformed prostate epithelial cell lines, these data suggest that the Gα12 proteins are up-regulated during prostate carcinogenesis. Interestingly, it appears that Gα12 is up-regulated at similar points in both breast (30) and prostate cancer development. In addition, it appears that this increase in Gα12 and Gα13 expression seen in prostate cancer parallels the increase in expression previously reported for the Rho GTPases (47, 48) and several of the GPCRs known to promote prostate cancer cell invasion and metastasis.
(5, 7, 8, 49). As such, it appears that as prostate cancer progresses, the \( G_{12} \) signaling pathway may be up-regulated as an invasion-promoting signaling unit.

This study also establishes the \( G_{12} \) proteins as potent stimulators of prostate cancer cell invasion. Although much of the work to date on the role of the \( G_{12} \) family of proteins in cancer has focused on the ability of these proteins to promote cell growth and transformation (18, 19, 34, 35), work in other systems has implicated a role for these proteins in cell migration. In addition to the study noted above demonstrating a role for \( G_{12} \) proteins in breast cancer metastasis (30), reports have appeared suggesting a role for these proteins in neutrophil (25) and fibroblast migration (50). Further, studies on the role of \( G_{12} \) signaling in development have suggested that genetic ablation of the \( G_{12} \) proteins results in impaired cell migration (21–24).

Interestingly, some of these studies that were conducted in *Drosophila* and zebrafish, revealed that signaling through the \( G_{12} \) family is required for the cell shape changes and movements required for normal gastrulation. Since many studies have demonstrated close parallels between gastrulation and cancer invasion (26–29), our findings that the \( G_{12} \) proteins are critical regulators of cancer invasion may represent the latest example of cancer usurping a normal biologic process to grow and metastasize.

Most of the studies demonstrating a role for \( G_{12} \) signaling in cell migration have focused on the ability of \( G_{12} \) and \( G_{13} \) to stimulate Rho. Moreover, a number of previous reports demonstrate that increased RhoA/C activity can promote invasion of prostate (48, 51, 52) and other cancers (41, 53–55). Thus, it was not surprising that \( G_{12} \)-induced Rho activation was strictly required for \( G_{12} \)-induced prostate cancer cell invasion. Since the invasion-promoting effects of the Rho pathway are pleiotropic and include such elements as activation of transcription factors, control of actinomysin contractility and cell polarity, modulation of cell adhesion, and induction of the epithelial to mesenchymal transition (41), it will now be interesting to determine which of these effects are important in \( G_{12} \)-induced invasion.

Previous studies from our laboratory have provided evidence that \( G_{12} \) signaling has the potential to promote cell migration in a Rho-independent manner. We have previously demonstrated that \( G_{12} \) and \( G_{13} \) are able to inactivate E-cadherin through a direct interaction with its cytoplasmic tail and promote cell migration in E-cadherin-positive cells (42, 43). Therefore, we were somewhat surprised that \( G_{12} \)-induced inactivation of E-cadherin was not sufficient to promote invasion of the DU145 cell line. Nevertheless, this result is consistent with a previous report demonstrating that inactivation of E-cadherin on DU145 cells using an antibody to block E-cadherin function resulted in only a minimal increase in cell migration (56). As such, the increase in cell motility resulting from the inactivation of E-cadherin by activated \( G_{12} \) (244–249) may not have been sufficient to produce a detectable change in DU145 cell invasion. Alternatively, these data may simply indicate that E-cadherin function has little involvement in prostate cancer cell invasion.
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We recently reported that the G12 proteins are up-regulated in breast cancer and that G12 signaling promotes breast cancer metastasis by stimulating breast cancer cell invasion (30). Together with the finding reported here that G12 proteins are up-regulated in prostate cancer and that signaling through this pathway promotes prostate cancer cell invasion, a view is emerging that the principle role of G12 signaling in these common human cancers is to stimulate invasion and metastasis and not tumor growth as was previously suggested. Further, from a clinical perspective, these studies suggest that targeted inhibition of G12 signaling may provide effective therapies to slow invasion and reduce the morbidity and mortality associated with these and possibly other cancers.

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