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DYSREGULATION OF GROWTH FACTOR-INDUCED MIGRATION IS RATE LIMITING FOR TUMOR CELL INVASION

by

JAREER KASSIS

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2001

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ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree PhD Program Pathology

Name of Candidate Jareer Kassis

Committee Chair Alan Wells

Title Dysregulation of Growth Factor-Induced Migration is Rate Limiting for Tumor Cell Invasion

The morbidity of solid tumors is predominantly due to tumor progression to the invasiveness. Tumor progression is strongly correlated with up-regulated signaling through the epidermal growth factor receptor (EGFR), which is the prototypal receptor that is responsible for a range of cellular signaling. Because one of the critical steps of tumor invasion has been postulated to be cellular migration and migration is one of the key processes modulated by the EGFR, we hypothesized that EGFR-mediated cell migration is key to tumor invasion.

One EGFR-mediated motility pathway is the PLCγ pathway. Inhibiting PLCγ delays invasion of EGFR-overexpressing cells through Matrigel. This is also true for ErbB2 overexpressing cell lines, which indicates that PLCγ is a convergence point for receptor-mediated migration and invasion. Transgenic mice expressing an inducible PLCγ dominant negative gene also have inhibited metastases of mammary tumors to the lungs, verifying the importance of PLCγ activity for tumor invasion. Another EGFR-mediated motility pathway that is independent of PLCγ is focal adhesion disassembly, in which calpain is a key molecule. Calpain inhibition also delays migration and Matrigel invasion of tumor cells. This indicates that EGFR-modulated invasion can be modulated

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by signaling via either mechanism. These data propose that migration per se, and not a specific single pathway, is rate limiting for invasion.

Invasion also involves proteomic changes as it occurs over time. The urokinase plasminogen activator receptor (uPAR) is the highest up-regulated gene in tumor cells engineered to by highly invasive via EGFR overexpression. Inhibition of uPAR with an antisense fragment reduces invasion through Matrigel and *in vivo*. These data demonstrate that uPAR overexpression is secondary to EGFR/PLCγ up-regulation and that invasion is a cooperative process where multiple pathways are stimulated to elicit the invasive phenotype.

Finally, in highly invasive cells in which PLCγ is not activated, invasiveness still correlates with increased migration, putatively through integrin signaling. Thus, in all studies, more invasive cells are necessarily more motile, regardless of mode of motility signaling. This establishes a solid conclusion that cellular migration through multiple receptor-mediated pathways is critical for tumor cell invasion.

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DEDICATION

This dissertation is dedicated to my parents,

Nabeel and Vanoush Kassis

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I extend my deepest gratitude to my mentor, Alan Wells, for his guidance, advice, and great support throughout my graduate career. The following members of the Wells Laboratory, past and present, were each invaluable in their support, collaborations, assistance, and friendship: Philip Chang, Jeffrey Chou, Kathryn Drabik, Mark Fung, Angela Glading, Kiran Gupta, Hyung-gyoon Kim, Han Lo, Jason Moellinger, Latha Satish, Hidenori Shiraha, James Solava, José Carlos Souto, Kate Sullivan, Scott Swindle, Kien Tran, Tim Turner, and Heng Xie. I also thank the members of my thesis committee, Drs. David Curiel, Ada Elgavish, Coral Lamartiniere, Carl Pinkert, and Eric Sorscher, each of whom provided valuable guidance and advice.

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A ROLE FOR PHOSPHOLIPASE C-γ-MEDIATED SIGNALING IN TUMOR CELL INVASION: SUPPLEMENT

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LIST OF ABBREVIATIONS

ACIF	Anticomplement immunofluoresence
AEBSF	4-(2-Aminoethyl)benzenesulfonyl fluoride
ATP	Adenosine triphosphate
BL	Burkitt Lymphoma
Boc-LM-CMAC	T-butoxycarbonyl-Leu-Met-chloromethylaminocoumarin
DAG	Diacyl glycerol
DPM3	Dolichol-phosphate-mannose-3
dFCS	Dialyzed fetal calf serum
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERK	Extracellular-signal regulated kinase
FAK	Focal adhesion kinase
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
GNRP	Guanine-nucleotide-release protein
GPI	Glycosyl phosphotidylinositol
GTP	Guanidine triphosphate

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HB-EGF	Heparin-binding epidermal growth factor
IGF	Insulin-like growth factor
IGF-IR	Insulin-like growth factor-I receptor
IgG	Immunoglobulin G
ILK	Intregrin-linked kinase
IP	Inositol phosphate
LTR	Long-terminal repeat
MAPK	Mitogen-activated protein kinase
MMTV	Mouse mammary tumor virus
NGF	Neuron Growth Factor
NPC	Nasopharyngeal carcinoma
p-Tyr	Phosphotyrosine
PAI	Plasminogen activator inhibitor
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PH	Pleckstrin homology
PI-3	Phosphatidylinositol-3
PIP_2	Phosphatidylinositol bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethanesulfonyl fluoride
PTB	Phospho-tyrosine binding

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RGD	Arginine-glycine-asparatate
RPTK	Receptor protein tyrosine kinase
TRAMP	Transgenic adenocarcinoma mouse prostate
SF/HGF	Scattor factor/hepatocyte growth factor
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
WASP	Wiscott Aldrich Associated Protein
WT	Wild-type

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INTRODUCTION

Invasive cancers are one of the major health problems worldwide and are projected to cause over half a million deaths in the United States alone in 2001 (1). While benign growths are usually curable, tumors that become invasive are considerably less treatable and often deadly (2). In order for tumor cells to acquire the invasive phenotype, they must first be able to recognize and attach to the extracellular matrix (ECM), then dissolve or rearrange the ECM, and lastly migrate through the modified ECM ectopically to adjacent host tissue (3). While each of these processes is highly significant and continues to be intensely investigated, it can be postulated that the third step, migration, is a rate-limiting step because it comprises an irreversible component of invasion. Migration is initiated or promoted by a multitude of processes that involves a highly intermingled network of signaling triggered by cell surface receptors. It is likely that some of these pathways become dysregulated during cellular transformation, causing or enhancing the aberrant migratory behavior that precedes invasion (4, 5). The studies presented in this dissertation lead to delineation of some of these pathways, which are generally considered to fall under mediation of receptor growth factor- or integrin-induced activity. Specifically, this dissertation presents a series of studies that elucidate key effecter and mediator pathways of tumor cell migration as induced by the epidermal growth factor receptor (EGFR) and its associated molecules.

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EGFR is a ubiquitous receptor. Apart from mediating vital cellular functions in normal conditions, it plays a significant role in the progression of cancers of various tissue types (6). Studies have shown EGFR to be overexpressed in such tumors as the breast, prostate, brain, bladder, colon carcinomas and glioma/glioblastoma. Irregular EGFR expression correlates with severity of progression and poorer patient prognoses (7-14). The receptor signals through various and often independent pathways and plays a vital role in cellular migration via a combination of such pathways (6, 15-17). In this introduction, the process of cell migration will be reviewed with emphasis on the mechanisms of action of EGFR and its associated molecules. The relevance of migration to enhancing tumor progression will also be discussed and placed in proper context to preface the experimental studies that follow.

CELL MIGRATION

Cell migration is an essential component of nonpathological biological events. For example, it is vital in embryonic development, especially during gastrulation when fibronectin-mediated tissue structure formation involves intensive cellular migration. Cell migration is also a component of response to injury, and inflammatory responses likewise involve movement of white blood cells into areas of insult to elicit their immunological actions. Wound healing depends on fibroblast and vascular endothelial cell migration into the injured area. Finally, migration is a principal promoter of tumor invasion, because it constitutes an irreversible penetration of tumor cells into adjacent tissue (5, 6).

The basic steps of cell migration are (a) polarization of the cell where the leading and trailing edges are clearly distinguishable, (b) leading edge (lamellipodial) extension

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to a clear area, (c) cell body contraction to shift the bulk of the cell to its new position, and (d) trailing edge detachment (4, 18). Each of these steps involves highly integrated processes and recruitment of specific molecules to relevant cellular locations. At least two of these four stages are directly investigated in the studies presented here, to establish the process of cell migration as rate-limiting for invasion.

Polarization. Prior to any migratory activity, a cell must become polarized, or form a distinct leading edge and a trailing edge (18). This process is highly dependent on the cellular surroundings and ECM composition (18) and not necessarily on a single chemotactic stimulus. A combination of growth factor signals, cytoskeletal changes, and intracellular localization of molecules defines the positions of the cell's leading and trailing edges. Polarization requires the redistribution of adhesion- and de-adhesion-producing molecules to the leading and trailing edges, respectively, which is an event that leads to primary lamellipodial protrusion. The efficiency of cellular functions that establish and maintain protrusion determines the overall cell migration rate, which is primarily dependent on two main factors: locomotion speed and directional persistence (18). Locomotion speed is defined as the actual velocity of cell movement, while directional persistence is defined as the extent of continuity of movement in a constant direction. These two aspects appear to be inversely correlated (19).

Membrane Extension and Adhesion. There are two main types of membrane protrusions that occur during migration: lamellipodia and filopodia (18). Both are devoid of organelles. Lamellipodia resemble broad, flat structures, whereas filopodia are more

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pointed and spindle-like. Generally, a series of filopodial membrane ruffles give rise to permanent extensions of the membrane, followed by adhesion to the extracellular surface (20, 21). Extensions are almost completely driven by actin polymerization (22, 23). Formation of the adhesion complex establishes a convergence of actin polymerization sites to the area, followed by highly active addition of monomeric G-actin to the F-actin complexes at proximal points to the membrane (18). A number of actin modifying proteins play a critical role at this juncture, including such players as cofilin and gelsolin, which function to sever F-actin, and profilin, which in turn modulates actin rearrangement (24). This membrane reorganization figures prominently in the underlying rationale for the first two manuscripts.

Membrane extension occurs in conjunction with formation of adhesion complexes, which is a receptor-mediated process (4). Of the multiple receptor families involved in stable cell attachment to the surface, the integrin receptor family is the best studied (25). Other receptor families are equally notable, including cell surface proteoglycans (e.g., syndecans and CD44) (26) and tyrosine kinase receptors [EGFR, platelet-derived growth factor receptor (PDGFR), insulin-like growth factor-I receptor (IGF-1R), and others] (6, 15, 27, 28). Strong adhesions commence with an active localization of adhesion receptors to the cell surface in the vicinity of the leading edge (29). Cytoplasmic domains of these receptors in turn recruit structural and adapter proteins as well as various signaling molecules, which act to create three-dimensional cytoskeleton-binding structures known as focal adhesions. These are spear-shaped (circatrix) structures of densely packed receptors, adapters, and signaling proteins that secure the ventral plasma membrane to the ECM (30, 31). Cytoplasmic structural proteins include talin, vinculin,

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and F-actin. Adapter proteins include Grb2 and Sos (32). Modification of proteins by tyrosine phosphorylation is a strong driving force for focal adhesion formation (29, 33), which is a critical step in migration (32). Indeed, there are a plethora of tyrosine-phosphorylated signaling proteins that promote this critical migratory step. Furthermore, tyrosine phosphorylation inhibitors significantly retard migration (34). Receptors at the cell surface have been shown to interact with tyrosine phosphorylated molecules such as focal adhesion kinase (FAK) (35), paxillin (36), phosphatidylinositol-3 (PI-3) kinase (37), Rho guanidine triphosphatases (GTPases) (38), and phospholipase C (PLC)γ (39). These molecules are organized into signaling clusters, some of which involve SH2 domain binding, as is the case for FAK signaling of csk and src (35) or paxillin binding of tensin (40). Thus, a complex network of interconnected signaling pathways directs the pace of migration. An important question pursued in this dissertation is whether such pathways are necessarily interdependent for their activation and, more importantly, if they are required for tumor cell invasion, either individually or in combination.

In addition to the signaling events that follow focal adhesion assembly, another type of cell membrane protrusion and membrane attachment is regulated by growth factors such as epidermal growth factore (EGF), neuron growth factor (NGF), and platelet-derived growth factore (PDGF), and their receptors (4). These can either supplement the signaling pathways initiated by the focal adhesion complexes or provide independent avenues of stimulation of extension and/or adhesion. For example, downstream signaling of EGFR or PDGFR elicits PLCγ activation (15, 28), which then cleaves membrane-bound phosphatidylinositol bisphosphate (PIP_2), releasing bound actin-modifying proteins into the cytoplasm (41). Regulation of release of these actin-modifying proteins by

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EGFR/PLCγ may correspond to regulation of cdc42, rac, and rho (38). The latter are members of a GTP-binding family linked to regulation of focal adhesion formation (42-44).

Cell Contraction and Traction. A migrating cell exhibits two types of forces: an actin polymerization-dependent protrusive force that extends the membrane and a contractive force that is driven by myosin motors inserting between actin bundles and which moves the cell forward (18). A balance of these two forces, which are related but not necessarily dependent on each other (45, 46), is necessary to achieve migration. Strength of adhesion varies across the leading edge depending on the molecular and spatial distribution of adhesive elements, and, separately, contractile forces within the cell are also manipulated by such factors as cell shape and substrate texture (18). Interestingly, the strength of these contractile forces feed back on the integrity of focal adhesions (i.e., inside-out signaling), because persistence and maintenance of focal adhesion structures depend on contractile force-induced accumulation of integrins to the focal adhesion sites (32, 47). Blocking contraction by inhibiting myosin light chain kinase results in integrin cluster disintegration and focal adhesion disassembly (48).

Extensive studies on cell contractility, primarily in Dictyostelium, have elucidated many vital aspects, not the least of which is the importance of myosin motors. Myosin I (a short-tailed single-headed protein) and myosin II (a longer-tailed, double-headed protein) are adenosine triphosphatases (ATPases) that move along the actin filament by utilizing ATP hydrolysis to affect conformational change (49). Myosin I is a monomer, while myosin II is generally found in dimer form, thereby providing it a double head at

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each end and thus the ability to attach to two actin filaments and affectively slide them past each other in contraction, following stimulation by calcium. Myosin II motors can effectively detach a cell from the surface depending on distribution and structural organization and, as such, may play a role in breaking adhesive interaction by inducing physical stress. This is evidenced by a concentration of myosin II-activating calcium at the trailing edge of some migrating cell types (50). Myosin I or II may also localize behind the leading edge but ventral to the nucleus. Thus two types of contractive forces may be functional; the first is just behind the leading edge to push the cell forward, and the other is at the trailing edge to facilitate dissociation from the substratum and relax adhesion-related resistance. It should be noted, though, that myosins are not required for migration, because myosin deficient cells still migrate, although more slowly (46, 51).

Rear Detachment. This final step of migration is, at least in some cell types, the determining factor of overall migration rate, as efficient motility entails timely release of the trailing edge of the cell from the substratum (45). Release of cell adhesions in the trailing edge can occur via mechanical stress due to contraction, as discussed previously, or stimulation of relevant signaling pathways. Incidentally, mechanical stress due to contraction is implicated in not only focal adhesion breakdown but also reorganization. Inhibition of cell contraction by mutating myosin II or inactivating myosin light chain kinase preserves focal adhesions and significantly retards migration (52), whereas overstimulation of Myosin activity via ATP supplementation increases the rate of adhesion breakdown in fibroblasts (53). Additionally, breakdown of adhesion complexes are subject to changes of focal adhesion component affinity to the substratum; i.e., integrins and

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other molecules that comprise the focal adhesion are "signaled" to shift affinity for the substratum negatively, independent of integrin density at the site (a process known as affinity modulation) (18, 54). Signaling pathways that are involved in this are under investigation, and one key player is thought to be the GTP-binding protein rho. Inhibiting rho results in reduced integrin clustering and focal adhesion disassembly, effectively causing cell rounding and cessation of motility (55, 56). Other players include the calcium/calmodulin regulated phosphatase calcineurin, which releases cells from vitronectin (57), and tyrosine kinases, which are thought to play a major role due to a significant rounding of cells and decreased adhesion when stimulated with ATP or tyrosine kinase encoder genes (31). Focal adhesion disassembly, with its vital role in cell migration, was targeted in the studies presented in the second manuscript of this dissertation as an independent signaling pathway from growth factor receptors to determine it is required for tumor cell invasion.

As focal adhesions are primarily composed of integrins, two possible fates for these molecules follow detachment: retention in the cell or depositing on the substratum (4). In the case of the former, integrin receptor affinity to the substratum components is diminished, allowing for separation to occur, while, for the latter case, tyrosine phosphorylation events are involved (58, 59). Integrins remaining on the cell surface are either endocytosed or actively redistributed and mainly recycled to the leading edge (58, 60). While the leading edge of the cell is primed for membrane extension and attachment to the substratum, the trailing edge is poorly supportive of these processes. Particularly, integrin association with cytoskeletal components, such as actin and its modifying proteins, does not occur at the trailing edge, and some regions of the trailing edge are alto-

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gether depleted of cytoskeletal components, which cause them to readily separate from the cell mass during migration (61, 62).

Overall Cycling of Migration Processes. It can be inferred from the above discussion that the overall rate of migration depends on efficient assembly followed by rapid disassembly of focal adhesions. Due to the delicate balance of the processes outlined above, it can further be inferred that maximal migration speed occurs at intermediate adhesion strengths, which allow attachment at the lamellipod but are still relatively easily detachable once at the trailing edge (18, 63, 64). Mechanical and chemical factors that determine adhesion strength and migration rate are carefully coordinated. Their relative roles almost certainly depend on cell type and environment. Furthermore, any abnormal phenotypes that occur in cases such as wound formation or oncogenic transformation are also likely to affect adhesion strength and migration rate. Focal adhesions are in the spotlight of migration, since it is largely the dynamics of their formation and disintegration that govern the overall process (32, 65). These adhesion points are by no means static clusters of molecules. Rather, they are highly active and are constantly being remodeled and resized in response to various stimuli that promote migration (66). Receptor signaling plays a vital role in the continued cycling of these processes.

The focus of this dissertation is the role of EGFR-mediated pathways in the promotion of cell migration, particularly in pathological conditions, namely tumor cell invasion. To better understand the role of these specific signaling pathways, the general scheme of motility signaling is addressed in the next section.

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ROLE OF INTEGRINS IN CELL MIGRATION

Numerous branches of cell migration signaling have been described (6, 67). The signaling pathways studied in this dissertation are generally attributed to integrin receptor signaling, detailed in this section, and/or growth factor receptor signaling, discussed in the next. As the aim of these studies is to elucidate the role of cell migration per se, this necessitates a careful description of both categories in order to intelligently decipher the overall findings, as well as elucidate any links that may be construed between the two systems. It is important to note that, despite seemingly independent roles for many of these signaling molecules and their modulation of often mutually exclusive pathways, a delicate harmony and balance exists in the activity of key players of these separate pathways. Such aspects will be further clarified in later sections, especially those relevant to the multifaceted EGFR.

Integrin Receptor Signaling. Despite their lack of intrinsic kinase activity, the importance of integrins in cell migration, as well as in invasion, is their double role of providing physical links to the ECM and mediating various signaling pathways that influence their adhesion and de-adhesion from the substratum (25). Integrins are heterodimers composed of a large α and small β subunit and are characterized by a large extracellular domain and a small cytoplasmic domain. Their functions are overlapping, i.e., multiple ligands can bind to a single receptor and vice versa. Integrins interact with their binding substrates via specific binding motifs, which commonly are short linear sequences containing an acidic amino acid residue (25). An example of this is the arginine-glycine-asparatate (RGD) binding motif (68), which was the first such motif discov-

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ered. It is found in fibronectin as well as other ECM molecules and growth factors. The integrin family spans a range of target-specific subgroups that bind different ECM components. For example, $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins are the major cellular collagen receptors, whereas integrins $\alpha 6\beta 1$ and $\alpha 7\beta 1$ are receptors for laminins (25). Focal adhesions are formed when integrins bound to their ligands relocate on the membrane to form clusters, although there are exceptions to this (25). Integrin recruitment to the focal adhesion sites relays further recruitment of other focal adhesion structural proteins such as tensin, talin, vinculin, and FAK. Tyrosine phosphorylation then ensues, which stimulates activity of such players as paxillin, mitogen-activated protein kinases (MAPKs), F-actin, and Src-type kinases (67). These signaling events are referred to as outside-in signaling, since they are initiated by ECM ligand binding to the integrin clusters (4, 25). Inside-out signaling, on the other hand, involves intracellular signaling that influences integrin affinity to its extracellular ligands. The exact manner in which this occurs is unclear but is postulated to involve integrin subunit conformational changes induced by intracellular signaling by serine/threonine kinases and small GTPases (69, 70). All these signaling events play a role in modulating the highly dynamic activities during integrin signaling of migration and other functions.

Integrin-mediated Tyrosine and Serine/Threonine Kinase Signaling. The most extensively studied tyrosine kinase signaled by integrins is FAK (71, 72). FAK signaling is involved in cell proliferation, apoptosis, ans migration, which is dependent on phosphorylation of residue Y^{397}. Both kinase activity and tyrosine phosphorylation of FAK are induced by integrins, specifically the β subunit's cytoplasmic domain (73-75).

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Phosphorylated FAK in turn activates its substrates, which include the SH2 domain-containing Src and PI-3 kinase proteins (35, 76, 77). A vital role for FAK in cell migration was demonstrated *in vivo* using FAK -/- knockout mice. This deletion was lethal in embryos, with the major defects occurring in vasculogenic and angiogenic processes that are dependent on cell migration (78-80). *In vitro* tests with cell cultured from FAK -/- mice also showed them to be less motile than cells from wild-type embryos. On the other hand, cells overexpressing FAK were more motile (81). The observation that activated FAK associates with other motility signaling proteins such as paxillin, talin, PLCγ, and PI-3 kinase (82-85) provides additional clues as to its overall role in migration. As described in later sections, many of these same molecules are major downstream signaling molecules to EGFR. The relevance of FAK to the studies presented here is seen in the fourth manuscript, where FAK activity is implicated as a growth factor receptor-independent derangement driving tumor invasion.

Src, whose family includes Hck and Fgr, is another integrin-regulated tyrosine kinase (86, 87). Src-related proteins are localized to focal adhesion in migration-stimulating conditions such as cell plating on fibronectin. Src-/- cells exhibit decreased spreading on fibronectin, whereas overexpression of Csk (of the Src family) causes decreased cell adhesion (86-88). Other tyrosine kinases modulated by integrins include the SH2/SH3 domain-containing c-Abl, which associates with paxillin, PI-3 kinase, and tensin (89-91) and whose activity correlates with adhesion, and Syk, whose phosphorylation correlates with integrin activity (92, 93). As tyrosine kinase proteins seem to play a role in integrin signaling, so do phosphatases, which by reversing the kinase signaling events play a major role in modulating the integrin signaling outcome. The phosphatase

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PTEN is one such widely studied phosphatase. It modulates phosphorylation of such key players as FAK and influences focal adhesion assembly and cell migration as a whole (94).

The Erk/MAPK family is a major serine/threonine kinase group regulated by integrin activity as well as by growth factor receptors, including EGFR (6, 95). Integrin activation in a number of cell types is followed by Erk phosphorylation (96, 97). Whether this process is dependent on Ras, which is an upstream activator of Erk, is still unclear (67). However, FAK may have a role since recruitment of Shc to phosphorylated FAK in turn promotes binding of Grb2, leading to Erk activation in some cell types (86, 98, 99). In other cell types there is a direct association of FAK with Grb2 (98). Integrin modulation of Erk activity may provide subdued yet constant mitogenic signaling, as opposed to rapid stimulation by growth factor receptors (67). Integrin-mediated Erk activity is also involved in cell spreading and migration (100, 101). Interestingly, integrin inactivation has been associated with Raf/Ras-promoted Erk activity, indicating a possible negative feedback mechanism of integrin regulation (67, 102). Further studies are required to better elucidate the details of the integrin-Erk/MAPK relationship. Integrin-linked kinase (ILK) is a ubiquitous serine/threonine kinase that binds directly to β1 or β3 integrins and is present in focal adhesion points (103). It is rapidly activated *in vitro* when plated on fibronectin, but its activity is diminished within 60 min (104). ILK also interacts with components of various signaling pathways and as such offers other avenues of integrin signal transduction (105). It has also been shown to be activated by PDGF and insulin and, interestingly, is inhibited by PI-3 kinase inhibitors (104), suggesting it is phosphatidylinositol-dependent. Downstream effects of ILK include manipulation of E-cadherin/β-

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catenin function, which is evidently due to phosphorylation of intermediate kinases such as GSK-3 (103).

Integrin Signaling of Phosphatidylinositol Lipids. PIP_2 is an active second messenger involved in regulating signal transduction of integrins and (as discussed later) growth factor receptors. PIP_2 binds actin modifying proteins such as profilin and gelsolin (106, 107) and as such manipulates the actin cytoskeleton and cell migration. Integrins activate PIP_2 most likely via intermediary proteins, which promote PIP_2 synthesizing molecules. For example, PI-3 kinase, an enzyme that phosphorylates the inositol ring, is recruited to the actin cytoskeleton following integrin activation and has further been shown to mediate cell migration secondary to integrin activity (108). Direct PI-3 kinase association with FAK has also been demonstrated and correlated with migration (109). Hence, activity of phosphatidylinositides such as PIP_2 is regulated by integrins through a variety of mechanisms.

Integrins and Rho Family GTPases. The Rho family of GTPases include Rho, Rac, and Cdc42. These molecules have been implicated in migratory cellular functions (67). Rho, a mediator of growth factor receptor signaling, modulates actin stress fibers and focal adhesion assembly. It is postulated to signal upstream of integrins (67). Evidence of this lies in studies that show integrins being a rate-limiting step for Rho-mediated signaling such as actin stress fiber formation or even focal adhesion formation itself, the latter also being mediated by Rac (110, 111). Further evidence supporting Rho as being upstream of integrins is that Rho activation increases FAK activation and tyro-

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sine phosphorylation, while Rho inactivation negates this effect (112). On the other hand, studies have shown that activated integrins trigger activity of PAK, which is a downstream affector molecule of Rac and Cdc42, suggesting that these GTPases signal downstream of integrins (113). Rac is implicated in lamellipodia and membrane ruffling via growth factor stimulation, while Cdc42 controls filopodial extensions likewise via growth factor receptor induction. The seemingly conflicting signaling sequences of integrins and Rho GTPases could be explained by suggesting a cyclical signaling pathway for these molecules as opposed to a linear mode of action (67). Alternatively, integrins and GTPases may perform complementary roles in such actions as focal adhesion formation, where evidence suggests that integrins are required for commencing focal adhesion assembly, while Rho GTPases are involved in completing the process (114). Since both sets of molecules influence cytoskeletal structure, this ultimately feeds back on integrins and GTPases themselves. Further studies are needed to clarify the intricacies of these events.

Integrin Association with uPAR. The urokinase-type plasminogen activator receptor (uPAR) is a pleiotropic chemokine involved in a variety of cellular functions (115). Its gene expression is manipulated by various effector molecules, implying a multifaceted role for this protein. The role of uPAR in cell migration and its association with integrins stem from its often required presence at the cells' leading edge for such functions as adhesion to occur (116-118). uPAR is also suspected of interacting with other focal adhesion proteins such as FAK (119). It has been shown to bind vitronectin as well as the integrin $\alpha v\beta 3$ vitronectin receptor (120). uPAR association with integrins has also been demonstrated by co-immunoprecipitation in keratinocytes and monocytes (121,

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122). Furthermore, a non-urokinase-type plasminogen activator (uPA) uPAR binding peptide has been isolated that modulates adhesion and integrin binding. This peptide is involved in induction of uPAR adhesion to vitronectin, as well as association with β1 integrins (123). Such findings implicate the uPAR system, which includes the ligand uPA and the plasminogen activator inhibitor (PAI)-1 and/or PAI-2, in adhesion and migration. Furthermore, association of uPAR with signaling molecules such as protein kinase C (PKC) and other tyrosine kinases (122, 124) points to its possible role in relaying cellular signaling events that influence the aforementioned processes. An important role for uPAR in cell migration is elucidated in this dissertation (third manuscript), because uPAR activity is linked to EGFR-induced migration at the gene expression level. This points to multifaceted avenues of activation for uPAR as well as linkage between growth factor motility signaling and invasion-promoting signaling.

GROWTH FACTOR REPECTORS AS MODULATORS OF MIGRATION

Growth factor receptors such as EGFR, PDGFR, and IGF-IR (i.e., receptor tyrosine kinases, RPTK) have been heavily implicated in cell migration (6, 27). While such receptors often converge on common downstream signaling pathways, they possess separate intriguing signaling characteristics. Here, an overview of key signaling processes that are modulated by growth factor receptors is presented. The molecules and pathways described are putative candidates for dysregulation, some of which are key to the findings reported in this dissertation. Growth factor receptors signal migration through many of the same pathways that regulate mitogenesis, although key motogenic pathways exist that are independent of mitogenic pathways (e.g., the PLCγ motility pathway) (15). Signaling

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via RPTKs can be categorized in separate groups. A first is the set of molecules that bind directly to the receptor's intracellular domains and are hence activated, and a second group consists of the downstream effector molecules that directly modulate the cytoskeletal events that produce migration. A third group, which has signals that influence gene expression that subsequently manipulate migration events, has also recently begun to be investigated, which an example of one of the key findings of the study presented in the third manuscript of this dissertation. The ensuing discussion, although generally applicable to RPTKs as a whole, will focus on the EGFR, which is the molecule of focus for the studies presented in this dissertation.

EGFR and Other Receptor Protein Tyrosine Kinases. The near ubiquitous 1186 amino acid EGFR (ErbB-1) is the prototypal member of the family of receptor tyrosine kinases (125). It is the best studied growth factor receptor not only due to its omnipresent nature but also due to the fact that it has a single isoform coded from a single gene on chromosome 7p11-13. The amino-terminal extracellular domain contains two cysteine-rich regions that form the ligand-binding domain. A single α helix comprises the transmembrane domain, followed by the cytoplasmic domain. An ~50 amino-acid region adjacent to the membrane forms a binding site for EGFR feedback inhibition molecules such as PKC and Erk/MAPKs. This is followed by the tyrosine kinase domain, an Src homology (SH)1 domain, whose activity has been shown to be required for cell migration. At the terminal end is the cross RPTK-conserved carboxy-terminal domain, which contains five tyrosine residues that bind SH2 or PTB (phospho-tyrosine binding) domains upon auto- or cross-phosphorylation (125). The carboxy-tail also contains inter-

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nalization and proteolytic activation sites. EGFR possesses specific ligands that do not overlap in activation of any other receptor; these include EGF, TGFα, amphiregulin, and HB-EGF (heparin-binding EGF-like ligand) (125). EGFR also interacts with and phosphorylates the three other members of the ErbB family (ErbB-2, 3, and 4), and all these receptors can hetero-aggregate (126, 127). Ligand binding to EGFR causes dimerization or hetero-aggregation of the receptors, activation of its kinase domain, and autophosphorylation of its tyrosine residues. The ligands of EGFR are able to stimulate the receptor through autocrine, paracrine, or juxtacrine methods (11, 128, 129). EGFR activity is down-regulated by receptor endocytosis, with its lifespan and ultimate fate (degradation or recycling) depending on the bound ligand (130). EGFR is present in all stromal and epithelial cells, as well as some glial and smooth muscle cells, and is associated among its myriad of activities with promoting cell migration.

Other similarly functioning growth factors receptors include the PDGFR family, which includes a triad of ligands (AA, BB, or the heterodimeric AB) (131, 132). Of the receptors themselves, PDGFR-β has been shown to become autophosphorylated upon ligand binding and is associated with chemotactic migration, whereas PDGFR-α is not (28, 37, 133, 134). This differential binding may regulate and specify PDGF-induced responses. c-Met is another tyrosine kinase, which, through binding of its ligand scatter factor / hepatocyte growth factor (SF/HGF) (135, 136), promotes cell motility in epithelial and endothelial cells. Recent evidence suggests cross talk between c-Met and EGFR in signaling of cell migration or invasion (137), which, aside from elucidating an interesting aspect of receptor cross-talk, also provides linkage of receptor tyrosine kinases to other avenues of cell migration, i.e., integrins and their signaling mechanisms, since HGF

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is also known to modulate uPAR activity (138). Insulin and IGF-I receptors are also known mediators of cell migration and chemotactic response (139, 140), in addition to metabolic actions.

Receptor-associated Molecules. EGFR and similar RPTKs have many common downstream binding substates, either by kinase-associated phosphorylation or binding and activation of affector molecules to the phosphorylated tyrosine residues. Most interaction of RPTKs with cytoplasmic molecules occurs via conserved domains that comprise the binding regions of these proteins. These include Src-homology-2 (SH2) domains, which bind short peptide motifs containing phosphotyrosine, Src-homology-3 (SH3) domains, which bind poly-proline sequences, PTB domains, which specifically recognize the concensus sequence NPXpY, and pleckstrin homology (PH) domains, which bind phospholipids or G-proteins (141-143). SH2 domains play the dominant role in RPTK signal transduction due to their phosphotyrosine recognition; binding via SH2 domains alters both physiological and morphological statuses of the molecules. Molecules that interact with RPTKs via SH2 domains are generally categorized as either signal transducers or adapter proteins. The former group comprises enzymes that catalyze activation or deactivation of other signaling molecules, whereas the latter group possesses no enzymatic activity but actively mediates recruitment of signaling transducers and enzymes to RPTKs.

Signal transducers associated with RPTKs. One of the immediately activated downstream molecules is phospholipase C-γ (39), which possesses two SH2 and one SH3

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domains and binds preferentially to Y^{992} on EGFR or Y^{1021} on PDGFR (144, 145). Recent studies have shown that inhibition of PLCγ suppresses migration in EGFR- or PDGFR-expressing cells but, at least in the case of fibroblasts and carcinoma cells, does not hinder mitogenesis (15, 146). It is also required for IGF-1- and HGF-induced motility (147, 148). Despite this requirement for motility, however, PLCγ is not sufficient for induction of migration (6). The role of PLCγ in migration can be attributed to its association with actin via its SH3 domain (149), as well as its enzymatic activity that cleaves its substrate PIP_2 to form diacyl glycerol (DAG) and inositol phosphate-3 (IP3), which separately modulate migration. PIP_2 cleavage also releases bound actin modifying proteins such as gelsolin or cofilin (107). PLCγ also activates PKC, which in some systems is involved in up-regulating chemotactic responses (150).

Another RPTK downstream effector molecule is PI-3 kinase, which is comprised of a catalytic and a regulatory subunit (151) and has also been shown to be required for motility in various cell systems (147). It also plays a significant role in integrin-mediated events. Cells expressing mutated PDGFβ receptors that are unable to bind PI-3 kinase lose chemotactic ability toward PDGF (37, 147), and PI-3 kinase inhibitors inhibit chemotaxis of both EGF- and PDGF-induced cells in certain systems (6, 27). Interestingly, evidence suggests that PI-3 kinase alone is insufficient for chemotactic responses in the absence of other molecules, including PLCγ; however, this has only been shown in limited systems (152). Another avenue of PI-3 kinase activity is its original role of phosphorylating phosphoinositides at the D-3 phosphate of the inositol ring, which in turn could activate PKC, rac, or PLCγ.

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The Src family tyrosine kinases bind via SH2 domains to RPTKs (153). While members of this family have no apparent effect on PDGF-induced signaling (6, 27), their role in EGFR signal transduction is still being deciphered. Src has an SH2 and an SH3 domain that are autoinhibitory; phosphotyrosine-bound SH2 domain causes conformational change that inhibits accessibility of Src substrates to its kinase domain (154). This autoinhibition is reversed upon Src binding to a phosphotyrosine residue of an RPTK, which renders the Src kinase domain susceptible to binding of its substrates. This mechanism, which has also been shown in Src family members Yes and Fyn, as well as Src protein (155), is considered the method of activation of Src by RPTKs. Src is presumed to interact with and modulate the assembly of the actin cytoskeleton (156, 157), and, furthermore, the Src SH2 domain binds the autophosphorylation site of FAK (158), which is another example of RPTK-integrin-focal adhesion interactivity. Further examples of this is the observation that Src activity correlates with that of Rac and Rho GTPases.

Stat proteins are a family of transcription factors that possess SH2 domains and have been shown to bind RPTKs (159, 160). They are traditionally implicated in cytokine/interferon receptor modulated signaling, which are constitutively associated with the JAK family of intracellular kinases. Ligand stimulation of these receptors results in JAK-associated tyrosine phosphorylation of these receptors and subsequent binding of a Stat family protein via its single SH2 domain. Stat phosphorylation then ensues, causing dimerization and translocation to the nucleus where transcriptional modulation occurs (161). The observation that Stat proteins bind to Y^{1068} and Y^{1086} of EGFR followed by

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coupling to JAK and translocation to the nucleus suggests a role for EGFR influence over transcriptional events as mediated by the JAK/Stat pathways (159, 160).

Adapter proteins associated with RPTKs. Adapters contain no enzymatic activity but do possess SH2 and SH3 domains. One such adapter protein is Grb2, which couples EGFR and other RPTKs to Ras (162, 163). Grb2 binds via its SH2 domain to Y^{1068} on the EGFR tail, which contains its consensus recognition sequence pYXNX (164). The SH3 domain of Grb2 subsequently binds to the Ras guanine-nucleotide-release proteins (GNRPs) mSos1 and mSos2, forming a tertiary EGFR-Grb2-mSos complex, which is required for Ras activation (162, 163). The SH3 domain of Grb2 can also bind other affector molecules such as dynamin, which is associated with clathrin-mediated endocytosis (165). RPTK association with Grb can also be indirect, as is the case with Shc binding RPTKs, becoming phosphorylated, and binding Grb2 via its SH2 domain (166). The tyrosine phosphatase Syp can also substitute for Shc in this action. Activated Syp is suspected of negatively regulating chemotactic response to PDGF (167, 168).

The adapter protein Nck-2 is of interest due to its role of bridging integrin- and growth factor-receptor signaling. Nck-2 binds the LIM domain protein PINCH, which is strongly associated with ILK (103). The Nck-2/PINCH/ILK complex is recruited via the NCK-2 SH2 domain to the phosphotyrosine residues of EGFR and PDGFR, providing an intriguing illustration of RPTK-integrin common signaling routes (169).

Downstream Signaling and Second Messengers. While the events that surround association of molecules directly with EGFR and other RPTKs are of vital interest, the

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effects of such interactions that occur downstream of these signaling-initiation events also bear greatly on the outcome of these signals and cell phenotypic changes. In this section, specific areas of relevance to this dissertation are discussed.

Ras signaling and mitogenesis. Ras is a vital element of EGFR- and other growth factor-induced signaling of cell proliferation as mediated by its activation of the MAPK pathway. It is a guanine nucleotide-binding protein, which renders it active when bound to GTP and inactive after it converts that to GDP via its intrinsic GTPase activity (162, 163). This GTPase activity is modulated by Ras-GAP, an SH2 containing adapter protein that is essentially a Ras inactivator molecule. In cells presenting mutated PDGFR that cannot phosphorylate Ras-GAP, chemotaxis was increased, indicating that Ras-GAP may be a negative regulator of migration (147). These cells had no discernible impairment of mitogenesis. The role of Ras itself in migration is controversial (6, 27). Ras activity is associated with migration of cells during wound repair in vascular endothelial cells (170), although its role in epithelial cells is less certain. Ras down-regulation does not necessarily impair cell movement in all cases, and it has been suggested that Ras is involved in cytoskeletal rearrangement but not movement per se (171). Furthermore, different Ras isoforms may be involved in inducing different cellular activities with respect to migration.

Kinases. Activation of the serine/threonine PKC family is strongly associated with EGFR signaling, but its role in migration is controversial (150). Activation of PKC by phorbol esters results in decreased growth factor-induced migration, whereas inhibi-

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tors of PKC kinase activity also cause reduction of migration (134, 172, 173). Since the classic isoforms of PKC which negatively regulate EGFR motility signaling are found in focal adhesion complexes, this kinase may mediate signals between growth factor receptor and adhesion receptor molecules, partially alleviating the controversy of its role (6).

The MAPKs are another group of downstream molecules that are instrumental in transducing RPTK signaling. Activation of Ras leads to the recruitment and activation of Raf (MAP kinase kinase kinase), which in turn signals MAP kinase kinase and finally activates MAP kinase (174, 175). Although studies show that MAP kinase is not sufficient for EGFR- or PDGFR-induced migration, its inhibition does abrogate migration (15). The Erk species of MAP kinases is activated by both integrins and growth factor receptors, and are implicated in myosin light chain kinase activation which leads to cell contraction (6, 100). They are also postulated to directly phosphorylate integrins, reducing their affinity to the ECM. By these mechanisms of induced contraction and rear detachment, Erk/MAPKs are implicated in focal adhesion disassembly, placing it in the category of rate-limiting for migration (95). Incidentally, focal adhesion disassembly as signaled by Erk/MAPK requires activity of the calcium-dependent proteolytic cleaver calpain (17). As these events appear to be independent of PLCγ signaling, it can be deduced that EGFR kinase activity and tyrosine autophosphorylation activity lead to independent signaling pathways. This is a vital premise of the studies presented here since determining the overall process of migration requires testing independent motility pathways.

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Rho family GTPases. These molecules have been implicated in EGFR signaling as well as integrin-mediated signaling. These molecules are molecular switches that modulate migration by influencing the actin cytoskeleton (38). Rac-induced membrane ruffling has been attributed to EGFR or PDGFR activity, as has Rho-induced formations of adhesions, although the sequence of signaling events relative to integrin and focal adhesion stimulation of these remains unknown. Growth factors may activate these GTPases secondary to Ras or PI-3 kinase. One study describes the transcription factor AP-1 as being required for EGFR activation of these GTPases in A431 cells (176). Due to the cyclical nature of receptors and possible nuclear elements that modulate activation of Rho, Rac, and Cdc42, it may not be possible to pinpoint the originating stimulus. However, one may attribute a certain process, for instance focal adhesion formation, as a trigger for GTPase activation.

The effects of downstream Rho GTPase signaling are also being investigated. They are obviously involved in alteration of the actin cytoskeleton as evidenced by their functions (38), but the exact mechanisms of how this occurs are elusive. It is possible that Rac interacts with actin modifying proteins such as gelsolin (177, 178), which it may actually activate via intermediary molecules such as PAK. Rho kinase, which is activated by Rho, is also involved in endothelial cell transmigration. Cdc42 activates the Wiscott Aldrich Associated Protein (WASP), which is involved in bundling high concentrations of polymerized actin (179). As is evident, there is yet much to be ascertained regarding the functions of these molecular switches.

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Second messengers. IP3 and DAG are two prominent second messengers that are the product of PIP_2 cleavage by PLCγ. IP3 induces intracellular calcium fluxes that promote calcium-dependent processes, including manipulation of actin assembly and activating various motility-promoting proteins (180). Separately, intracellular calcium levels have indeed been shown to be increased during locomotion or chemotaxis (181, 182), with a major role for RPTKs described (28, 183). Calcium gradients caused by these fluxes may influence cellular location of actin assembly and disassembly and thus enhance forward movement (184, 185). DAG is a potent activator of PKC, whose role in migration is referred to earlier, and is also directly associated with actin filament assembly with the ability to increase the rate of assembly in the presence of a plasma membrane by promoting formation of actin nucleation sites. These effects provide additional mechanisms of RPTK-mediated cytoskeletal modulation.

Actin modifying proteins. Another result of PIP_2 cleavage by PLCγ is the release of PIP_2-bound actin modifying proteins such as profilin or gelsolin. Profilin is a 15,000-MW protein, which binds ATP-actin monomers and also facilitates exchange of ADP for ATP on these monomers, the latter being required for polymerization and elongation to occur (23, 186). Although profilin itself binds and sequesters actin monomers, its properties suggest that such sequestering is part of a more elaborate polymerization mechanism. Gelsolin is an 87,000-MW protein that binds to and severs polymerized actin filaments (187). Since both profilin and gelsolin are inhibited by their anchorage to PIP_2, their release through PLCγ activity is an important event in actin filament assembly and subsequent cell motility.

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TUMOR CELL INVASION AS DYSREGULATED MOTILITY

Cell migration, with its plethora of intricately woven signaling pathways, is the final step of invasion, the first two being recognition and attachment to the ECM, followed by dissolving and reorganization of the ECM. Obviously, it is unfeasible to pinpoint a single irregularity in the process of migration that would trigger the invasive phenotype. Rather, it is more plausible to suggest that migration-induced cell invasion requires an overall phenotypic and often genotypic change in various cellular migration mechanisms. As a result of these changes, the usual four-step mechanism of migration, outlined early in this introduction, may not be strictly followed in the event of transformation to an invasive phenotype (4).

Most events that promote invasion involve hyperactivity of signaling elements of migration. Increased expression of integrins, such as $\alpha v\beta 3$ or $\alpha 5\beta 1$, appears to correlate with higher invasive and metastatic potential. Dysregulation of many of the effector molecules described for motility can potentially influence invasion of transformed cells (5). The focus of this dissertation is growth factor receptor dysregulation, specifically EGFR dysregulation, in tumor cell invasion. The manuscripts that follow describe studies that implicate or widen the understanding of the role of EGFR and its signaling pathways in tumor cell invasion. The first aim of these studies was to establish a proof of concept that EGFR-mediated signaling of migration is rate-limiting for invasion. After establishing this model, the next step involved describing the molecular players that are involved in cellular migration and demonstrating their role in tumor cell invasion by observing the effects of their inhibition. A central question to these studies was whether a specific motility-inducing pathway could be pinpointed as rate-limiting for invasion or if the

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process of migration as a whole is rate-limiting, which was a study performed by targeting independent avenues of migratory stimuli. Upon determining the answers to this, there remained the question of what secondary changes, particularly at the genetic level, would accompany the observed phenotypic changes that are responsible for invasion. This question was answered by comparing gene regulation of tumor cells that were engineered to migrate at different rates. Finally, the notion of there being an "exception to the rule" was analyzed by testing a system where the generic migratory pathways described earlier were not active, although integrin-mediated events did appear to be taking place. Even in this case, evidence still showed that migration is rate limiting for invasion albeit via unconventional signaling modulators. The results of these studies are outlined in the manuscripts that follow, concluding with a concise analysis of the discoveries set forth in this study within context of cellular migration and invasion as understood to date.

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DISSERTATION OVERVIEW

MANUSCRIPT 1: A ROLE FOR PHOSPHOLIPASE C-γ-MEDIATED SIGNALING IN TUMOR CELL INVASION

The introduction of this dissertation previewed the general mechanisms of cell migration and speculated on the role of migration in tumor cell invasion. The focus of the studies presented here was one of the main promoters of migration, i.e., growth factor receptor-mediated motility as represented by EGFR, and its role in cell invasion. The aim of this paper was to describe an initial multitissue type model that establishes the definite concept of growth factor receptor-modulated cell migration being a rate-limiting step for tumor cell invasion. Previous studies verified a vital role for PLCγ, one of the SH2 domain-containing downstream molecules from RPTKs, as required for EGF-induced motility. Our initial studies established that, in a single prostate cancer cell line, DU-145, inhibition of PLCγ significantly reduced invasion of these cells both *in vitro* and *in vivo*. In this paper, we asked whether this role was specific to one type of prostate line or more universal. Using tumor cells derived from a transgenic mouse model (TRAMP) as well as breast cancer cell lines, we found that inhibiting PLCγ with the PLC inhibitor drug U73122 reduced invasion of these cells through Matrigel. Also, stably expressing a PLCγ dominant negative, PLCz, in a breast cancer cell line inhibited its invasion through Matrigel. All these cells expressed up-regulated EGFR or ErbB2 that signaled in an autocrine manner, and PLCγ activity followed EGF treatment in the EGFR expressing lines and was constitutively active in ErbB2 expressing cells. That PLCγ signaling was

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secondary to EGFR receptor signaling was shown by blocking EGFR kinase activity, which inhibited both PLCγ phosphorylation and Matrigel invasion in both TRAMP and MDA-468 cell lines. Thus, RPTK signaling of PLCγ is introduced in this first manuscript as being a key element in promoting tumor cell invasion.

MANUSCRIPT 2: MOTILITY IS RATE-LIMITING FOR INVASION OF BLADDER CARCINOMA CELL LINES

The aims of the studies in this manuscript were two-fold. The main goal was to answer the question of whether the observed inhibition of invasion by hindering PLCγ activity is an event specific to that pathway or to the process of motility as a whole, of which PLCγ is but one key molecule. A subordinate goal was to assess the validity of the findings of manuscript 1 in a new system, aggressive bladder carcinoma. A previously untested metastatic cell line, 253J-BV bladder carcinoma, was used. This cell line expresses high levels of EGFR, and PLCγ is phosphorylated by EGF, although it exhibits high background phosphorylation due to autocrine signaling. In these cells, inhibiting PLCγ, either pharmacologically with U73122 or by expressing the PLCz dominant negative, resulted in significantly decreased Matrigel transmigration. As described in the introduction, another arm of cell migration is rear cell detachment as modulated by focal adhesion disassembly. This mechanism is dependent on MAPK activity and was recently shown to involve calpain, which has surfaced as a pivotal molecule in cell migration but is independent of PLCγ. In this manuscript, it was shown that inhibition of calpain significantly reduced 253J-BV cell invasion through Matrigel. These studies showed that, by inhibiting migration through separate arms, it is strongly implied that it is migration per se and not a particular pathway such as PLCγ that is rate-limiting in tumor cell invasion.

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The results of this study provided an important clue to the process of migration as a whole. The evidence shown here suggests that, since migration is a multifaceted process, it would appear that the many separate branches of migration, as described in the introduction, are collectively required to produce an overall phenotypic change. Furthermore, it can be concluded that a rate-limiting step for invasion appears to be the process of migration as a whole and not a single migration-promoting pathway in particular.

MANUSCRIPT 3: INVASIVENESS OF DU-145 HUMAN PROSTATE CARCINOMA CELLS EXPRESSING DIFFERENTIALLY ACTIVATED PLCγ INVOLVES ALTERED uPAR EXPRESSION

Invasion occurs over weeks to months, a period during which proteomic changes can occur. The question arose whether gene expression changes contribute to invasion. Previous studies had established sublines of the invasive DU-145 prostate cancers cells that were engineered to differentially activate PLCγ; DU-145 cells that overexpressed EGFR exhibited significantly higher invasiveness *in vitro* and *in vivo*, while another subline that contained a truncated EGFR that is fully mitogenic but does not signal PLCγ was found to be relatively uninvasive. These sublines were ideal for this study.

DU-145 sublines [highly invasive wild-type (WT) EGFR, moderately invasive parental, and non-invasive c'973 EGFR] were subjected to microarray analysis. mRNA from all sublines were tested for variation of any of 9,216 candidate genes. The list of genes that were up-regulated in invasive DU-145 WT cells and down-regulated in DU-145 c'973 cells (deemed invasion promoter genes) was topped by uPAR, which has been implicated in advancing tumor invasion in many separate investigations. This finding was significant since it established a causal link between PLCγ up-regulation and activity of

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uPAR, which is a separate tumor-enhancing gene. In order to establish the importance of uPAR expression in modifying the invasive phenotype of cells that differently regulate PLCγ, a uPAR cDNA fragment driven by the constitutively active SV40 early promoter (pXf-uPAR) was constructed. This construct was stably expressed in DU-145 parental cells, and their invasiveness was tested through Matrigel. DU-145 parental cells expressing pXf-uPAR were significantly more invasive than their parental counterparts. In contrast, a pXf-antisense uPAR construct was stably expressed in highly invasive DU-145 WT cells. In this case, DU-145 WT cells expressing the antisense construct showed markedly reduced invasion. These studies were also performed in athymic nude mice; DU-145 WT cells were extremely invasive in mice as measured by diaphragm invasive, whereas DU-145 WT cells expressing antisense uPAR were significantly less aggressive *in vivo*. Hence, the studies described in this paper describe causality; enhanced signaling of EGFR via PLCγ caused up-regulation of uPAR, which separately modulates migratory events that are integrated with integrin activity and are concentrated at the leading edge. These results establish the concept of linkage between independent migration-inducing pathways as summarized in the introduction, although the results are not an attestation of dependency of signaling pathways on one another.

MANUSCRIPT 4: EBV-EXPRESSING AGS GASTRIC CARCINOMA CELL SUBLINES PRESENT INCREASED MOTILITY AND INVASIVENESS

This manuscript specifically addressed the possibility of whether cell migration in tumor invasion can be EGFR/PLCγ independent in specific circumstances, in an attempt to determine whether, even in the absence of previously defined specific migration pathways, invasion is still driven by cell motility. In other words, a cell system that promotes

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cell invasion without the direct involvement of one of the migration-inducing pathways, the PLCγ pathway, was analyzed to show that it is still migration as a cellular function per se that is the driving force behind tumor cell invasion.

A gastric carcinoma cell line (AGS) was obtained that had been infected with the Epstein-Barr Virus (EBV). EBV-infected sublines were observed to be significantly more invasive through Matrigel compared to parental AGS cells. *In vitro* migration assays on the sublines were performed to find that the more invasive EBV-infected cells were indeed more migratory than the parentals. However, further analysis of these cells showed undetectable levels of phosphorylated PLCγ across all the sublines, indicating that PLCγ may not be involved in enhanced migration and invasion of these cells. Treatment of these cells with various inhibitors of EGFR or PLCγ activity showed insignificant changes in migration rates observed. Therefore, we established that EBV-infected cells were more migratory and also more invasive through an EGFR/PLCγ independent mechanism. In order to evaluate which signaling pathways may be responsible for enhanced migration in the EBV-infected AGS cells, AGS sublines were tested for differential expression of integrin-related proteins since the common growth factor receptor convergence point, PLCγ, seemed not to be involved. No significant changes in the levels or activities of integrin αv or α4 or of paxillin, ILK, and MAPK were found. However, there was a significant increase in phosphorylation of FAK in the more migratory and invasive EBV-infected cells. These results indicate that specific (though PLCγ-unrelated) signaling pathways involving FAK and as yet unidentified integrins are putatively the main driving force behind invasion-promoting cell migration in these cells. The significance of these findings is not necessarily that PLCγ activation is not always required for

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migration and invasion; this assertion cannot be made because of the presence of exogenous virally encoded proteins with uncertain functions. Rather, these results indicate that, even in the absence of a conventional migration-inducing pathway (i.e., PLCγ), cells that exhibited increased Matrigel invasion were also more motile, again sustaining the overall hypothesis that migration as an overall process is a driving force of tumor cell invasion.

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A ROLE FOR PHOSPHOLIPASE C-γ-MEDIATED SIGNALING IN TUMOR CELL INVASION

by

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ABSTRACT

The invasive and metastatic transformation of cancers often results in death. However, the mechanisms that promote this remain unclear. Two closely-related receptors, the epidermal growth factor receptor (EGFR) and ErbB2, are overexpressed in a significant percentage of breast and prostate carcinomas among others, with this up-regulated signaling correlating with tumor progression. Previous studies in our lab have demonstrated that an EGFR-phospholipase C (PLC)γ-mediated motility-associated signaling pathway is rate-limiting for tumor cell invasion *in vitro* and *in vivo* in one model of prostate carcinoma. Therefore, we investigated whether this PLCγ signaling pathway also was rate-limiting for invasion in other tumor cell lines and types and whether this EGFR activity is subsumed by the closely related ErbB2. We determined the effects of PLCγ signal abrogation by pharmacological (U73122) and molecular (expression of the dominant-negative PLCz) means on the *in vitro* invasiveness of tumor cells. Inhibition of PLCγ signaling concomitantly decreased invasiveness of *de novo*-occurring transgenic adenocarcinoma mouse prostate (TRAMP) lines and the human breast cancer cell lines MDA-468 and MDA-231; these lines present up-regulated EGFR signaling. Because the prostate and breast cancer lines usually present autocrine stimulatory loops involving EGFR, we also examined transgenic adenocarcinoma mouse prostate C1 and MDA-468 treated with the EGFR-specific kinase inhibitor PD153035 to determine whether invasiveness is dependent on EGFR signaling. PD153035 reduced invasiveness to levels similar to those seen with U73122, suggesting that the autocrine EGFR stimulatory loop is functioning to promote invasiveness. To determine whether this signaling pathway also promotes invasiveness of ErbB2-overexpressing tumors, we examined the human breast carcinoma line MDA-361; again, U73122 inhibition

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of PLCγ decreased invasiveness. In all situations, the inhibition of PLCγ signaling did not decrease mitogenic signaling. Thus, the motility-associated PLCγ signaling pathway is a generalizable rate-limiting step for tumor cell progression.

INTRODUCTION

Cancers of the breast and prostate are among the leading causes of death in adults, collectively responsible for over 80,000 deaths annually in the United States (1). Although localized tumors are curable by excision and have good prognoses, invasive and metastatic tumors are often nonresponsive to treatments and present significant morbidity and mortality. Many different therapeutic treatments have been employed in the past years with only limited success because the exact mechanism of disease progression that ultimately causes death remains unclear. Therefore, elucidation of the mechanism of breast and prostate tumor invasion and metastasis would provide for future rational therapeutic approaches.

EGFR family members are significantly amplified and/or overexpressed in a wide variety of tumors. Specifically, both EGFR and its relative, ErbB2 (also referred to as neu and HER2), have been shown to be overexpressed in breast and prostate cancers. These cancers often express autocrine stimulatory loops involving EGFR and its ligands (2-5). Increased expression of EGFR and/or ErbB2 correlates with greater tumor invasion and metastasis and poorer prognosis in many cancers, including those of the breast and prostate (6-9). This observation invites the hypothesis that EGFR and ErbB2 play significant roles in tumor progression to the invasive and metastatic state.

There are a number of cellular mechanisms associated with tumor invasion and metastasis, including recognition of the extracellular matrix, protease activity that creates a

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defect through the matrix, and tumor cell migration. Among these, tumor cell migration is promoted by EGFR signaling and subsequent activation of PLCγ (10). Our previous studies have defined an EGFR-PLCγ signaling pathway that is required for enhanced cell motility but not mitogenesis (10, 11). In one prostate cancer cell line, DU-145 (12), we demonstrated that pharmacological or molecular inhibition of PLCγ signaling reduced cell invasiveness both *in vitro* and *in vivo* (13-15). The diminished invasiveness was attributed to decreased motility, based on biophysical studies in fibroblasts and the fact that abrogation of PLCγ signaling did not reduce cell proliferation or increase tumor cell apoptosis. Interestingly, PLCγ, which is activated by many growth factor receptors, including ErbB2 (16-20), has been shown to be required for motility signaling from a number of growth factor receptors, including those for platelet-derived growth factor and insulin-like growth factor-I (21, 22). We propose that this key molecule serves as a point of convergence that is utilized by diverse growth factors and, thus, may be a common target for tumor progression promoted by amplification of receptors in addition to EGFR, such as ErbB2. Thus, it is important to determine whether the concept of PLCγ-mediated migration being required for invasion is universal, which in itself would be an important contribution to the study of invasion and metastasis in malignancies.

To determine whether the role of EGFR as observed in DU-145 is generalizable, we investigated the PLCγ motility pathway in other cells derived from prostate and breast carcinomas. The prostate line chosen is from a mouse prostate tumor which occurs *de novo* in the TRAMP model (23, 24). These mice express the SV40 large T-antigen driven by a probasin promoter. Furthermore, because these mice develop invasive prostate carcinomas, using these cells would enable the extension of any finding to *in vivo* settings in the future.

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To determine whether PLCγ-mediated signaling was important in the progression of other tumors arising in sex hormone responsive tissue, breast cancer cell lines were examined. Because EGFR overexpression has been correlated with tumor progression and poor metastasis in large series of breast carcinomas (8, 25, 26), the EGFR-overexpressing lines MDA-468 and MDA-231 were chosen. However, amplification of c-erbB2/neu also is frequent in metastatic breast carcinomas (9, 27). Because this closely related EGFR family member also presents PLCγ docking sites and activates PLCγ (28-32), we postulated that erbB2 signaling may subsume a similar role as EGFR signaling and requires the proposed convergent PLCγ signaling pathway for increased tumor invasion. Thus, the role of PLCγ signaling in the invasiveness of an erbB2 overexpressing line, MDA-361, was investigated. In short, we found that pharmacological and/or molecular inhibition of PLCγ reduced invasion of these cell types through Matrigel and that inhibition of EGFR kinase disrupted an autocrine stimulatory loop driving this invasion. These findings suggest that growth factor-induced motility involving PLCγ signaling is generalizable to a variety of tumor types and may serve as a target to limit tumor progression.

MATERIALS AND METHODS

Cell Lines. TRAMP C1 and TRAMP C2 cells were derived from prostate tumors that developed in the TRAMP (23, 24). The cells were maintained in high-glucose DMEM containing 10% FCS and supplemented with insulin (5 ng/ml), penicillin (25 units/ml), streptomycin (25 μg/ml), and dihydrotestosterone (10^{-8} M). MDA-MB-468 (MDA-468) were a kind gift from Dr. Jeffrey Kudlow (University of Alabama at Birmingham, Birmingham, AL), whereas MDA-231 and MDA-361 cells were obtained from the American Type

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Culture Collection. The breast cancer MDA cell lines were maintained in DMEM containing 10% FCS supplemented with L-glutamine (2 mM), penicillin/streptomycin (100 units/ml), non essential amino acids (0.1 mM), and sodium pyruvate (1 mM). Media for the MDA-361 cells also contained 10 ng/ml insulin as recommended previously (33).

TRAMP Characterization By RT-PCR and Immunoblotting. RNA was isolated from both TRAMP C1 and TRAMP C2 by the Trizol method (Life Technologies, Inc.; catalog #15596-018). Suitable primers were designed in accordance to DNA maps of EGFR, EGF, and TGFα in which ~50 bp of each map was selected based on minimal looping, suitable G-C:A-T ratio, and the presence of one restriction site within the fragment for confirmation. RT-PCR was performed on RNA isolated from both TRAMP C1 and TRAMP C2 (primers for EGFR: 5'-AGACCATCCAGGAGGTGGC, 3'-GATGGCTCTGTAAGTCCATTG; for EGF: 5'-TTGCCCTGACTCTACCGCAC, 3'-CCACCATGATGTCATGCTTCTG; and for TGFα: 5'-GGTGCAGGAAGAGAAG-CCAG, 3'-GCACGGCACCACTCACAGTG) for 30 cycles. PCR products were run on 1% agarose gels both intact and cut with the appropriate restriction enzymes to confirm the identity of the bands. For immunoblots, cell lysates were obtained from confluent cells, size-fractionated, and analyzed as below. These were then probed with antibodies that recognize the murine EGFR (Transduction Laboratories; catalog #12020) and murine pro-TGFα (a kind gift from Dr. Jeffrey Kudlow; Ref 34). Membranes exposed to the the pro-TGFα antibody were blocked in 5% fat-free milk containing 100 μM DTT, and the antibody itself was diluted in TBS (10 μg/ml) with 0.05% Tween-20 and containing 5 μM DTT.

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Expression of Dominant-Negative PLCγ Fragment PLCz. Introduction of exogenously encoded PLCz was accomplished using the lipofectin method. PLCz is a dominant negative fragment of PLCγ and consists of the SH2 and SH3 domains and an inhibitory (I) domain (35). The constitutively expressed PLCz (pXf PLCz), as well as its control (pXfcontrol), was transcribed from an SV40 early promoter. Briefly, the DNA (5-10 μg) was precipitated in ethanol and 0.25 M ammonium acetate and then mixed with 30 μl lipofectin (Life Technologies, Inc.) before introduction into 6-well plates containing MDA-468 cells. Media was replaced after 5 h, and transfected cells were selected in media containing 1200 nM methotrexate. Expression was verified by immunoblotting with an anti-PLCγ antibody.

Immunoprecipitation/Immunoblotting. Protein expression and phosphorylation were determined as previously described (10). Cells were tested under conditions that either enable or minimize paracrine/autocrine signaling. To observe the autocrine signaling, cells were grown to confluence and then maintained at maximal confluence for at least 48 h in media containing10% FCS in a 10-cm dish, after which cells were either lysed or incubated for a further 24 h under serum-free conditions before lysing. To minimize autocrine signaling, subconfluent cells were quiesced in 1% dialyzed FCS (dFCS) or serum-free media for 24 h, as determined for each cell line by basal thymidine incorporation and cell viability, followed by stimulation with EGF for 5 min. Cells were washed with PBS and lysed in lysis buffer [10% glycerol, 1% Triton X-100, 100 nM NaCl, 20 mM HEPES (pH 7.4), and 1 mM sodium vanadate] for 1 h at 4°C. After clarification by microcentrifugation, the lysate was incubated for 1.5 h with a specific antibody-agarose bead mixture that had

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been incubated overnight at 4°C. Primary antibodies included anti human EGFR (Oncogene Science; catalog #GR01), anti human ErbB2 (Calbiochem; catalog #OP39), and anti-PLCγ antibody (Upstate Biotechnologies, catalogue #05-163). The lysate-antibody-agarose bead mixture was then washed five times with lysis buffer prior to analysis by reducing SDS-PAGE and immunoblotting.

Transmigration Assays. Cell invasiveness *in vitro* was determined by the ability to transmigrate a layer of extracellular matrix, Matrigel, in a modified Boyden chamber assay (36, 37). Matrigel invasion chamber plates were obtained from Becton Dickinson/Biocoat (catalog #40480 and #40481). For each individual cell line, cells were plated randomly and distributed among plates with different lot numbers, with each experiment performed in triplicate. Despite possible variances in EGFR ligand concentrations in Matrigel, these concentrations are saturating because even "reduced growth factor Matrigel" contain relatively high amounts of EGF (up 0.5 ng/ml compared to 0.5-1.3 ng/ml in regular matrigel; Becton Dickinson Labware 1997/98 catalog description, page 128). Cells were kept in serum-free media containing 1% BSA for the first 24 h and then replaced with only serum-free media for the remaining 48 h. Enumeration of the cells that invaded through the matrix over a 72-h period was accomplished by two different methods. Initially, cells were metabolically labeled in the presence of 5 μCi/ml [methyl-[^{3}H]] thymidine and the acid-precipitable counts on the bottom of the filter and in the targeting well measured; this procedure eliminates cell proliferation as a confounding variable (15). The radioactivity associated with the well and on the bottom of the filter were consistent so that in later experiments only the filter-associated label was measured. In later experiments we visually counted cells

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on the bottom of the filter, as per routine procedures (36), after we verified that cell number corresponded with transmigrated radioactivity. In all cases, individual experiments were performed in triplicate.

Mitogenesis Assays. Cells were plated into 12-well plates, allowed to grow to confluence, and then placed in serum-free media for 48 h. After this, appropriate wells were treated with 10 nM EGF and/or appropriate concentrations of U73122 for an additional 18 h. [methyl-[^{3}H]] thymidine (5 μCi/ml) was added to each well, and incubation resumed for another 8 h. Wells were then washed with PBS, followed by acid precipitation (5% TCA, 4°C for 30 min). The wells were washed with PBS and then treated with 0.2 N NaOH to solubilize the incorporated radiolabel. Scintillation counting quantitated the amount of incorporated thymidine. Each experiment was performed in duplicate.

PLC Activity Assays. PLC activity was determined by measuring accumulation of IPs (38, 39). Cells were labeled by culturing in 5 μCi/ml myo [1,2-[^{3}H](N)] inositol for 24 h, after which the plates were washed twice with 37°C PBS. To limit degradation of inositol phosphates, 10 mM of lithium chloride was added to the cell media and incubated for 10 min before proceeding. The plates were then treated with 10 nM EGF for 30 min. Cells were lysed with 1 ml boiling water, and lysates were separated with a Dowex (AG1-X8 100-200 mesh) anion-exchange mini column containing 1 ml of 1:1 ionized sephadex resin:water to bind charged phosphate groups. The contents were eluted with buffers in the following sequence: water to release free inositol, sodium borate/sodium formate (5 mM/60 mM) to release glycero-phosphoinositol), and ammonium formate/formic acid (200

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mM/100 mM) to release inositol phosphate. The amount of IP was measured by obtaining radioactive counts of the final eluate. We have previously determined that this assay correlates closely with measuring IP3 production directly by more time-consuming high-performance liquid chromatography analysis (11). Furthermore, the extended time period of 30 min allows for significant accumulation of IP (in the presence of LiCl) and, thus, measures PLC-mediated turnover as a sensitive readout instead of steady-state IP3 (38, 39).

Statistical Analyses. All analyses were performed as paired Student's *t* tests, with a level of significance assigned at < 0.05.

RESULTS

EGFR-PLCγ Signaling Pathway Is Functional in Prostate and Breast Cancer Cell Lines. The TRAMP cell lines were derived recently, and their EGFR expression status was unknown. Using RT-PCR, we detected expression of EGFR and its primary ligands EGF and TGFα, indicating the possible presence of an autocrine loop in these cells (Fig. 1). Protein lysates of TRAMP C1 and TRAMP C2 cells were immunoblotted with antibodies generated against murine EGFR or murine pro-TGFα (because the cell lysate and not supernatant is probed). Both of these were recognized in TRAMP C1 and TRAMP C2 cells. Thus the TRAMP prostate cell lines present a potential autocrine stimulatory loop present in prostate epithelial and carcinoma cells. (We could not detect EGF due to the inability to obtain a suitable antibody against this ligand.)

To determine whether the cells under investigation in this study possess functional EGFR, cells were plated under conditions that minimize paracrine/released autocrine

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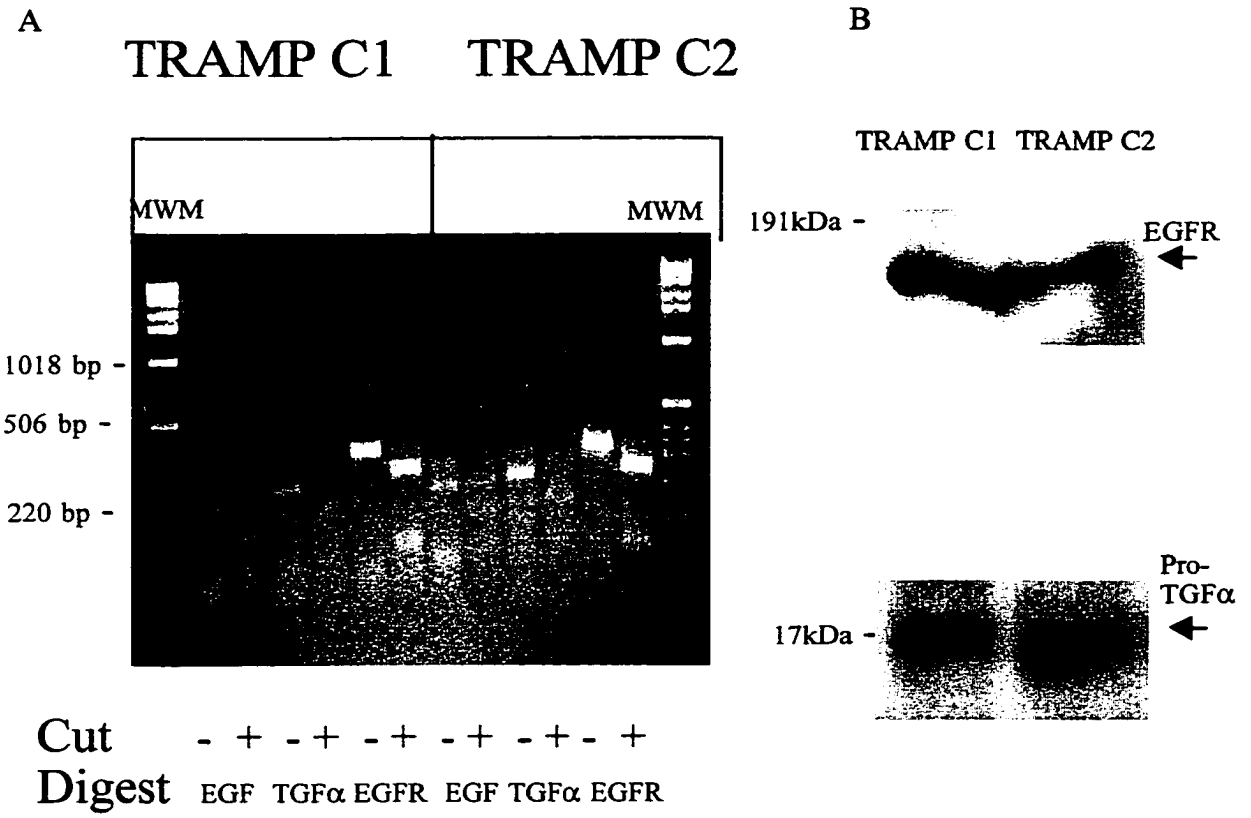

Fig. 1 Expression of EGFR and cognate ligands in TRAMP cell lines. *A*, RT-PCR of TRAMP C1 and TRAMP C2 cells was used to detect transcripts for EGFR and its ligands EGF and TGFα. Primers were constructed to recognize sequences within each of EGFR, EGF, and TGFα. *, bands of predicted size. Identification of these bands as specific for the target was verified by endonuclease digestion [*Cut* with *Sph*I (EGF and TGFα) or *Pst*I (EGFR)], yielding new bands of expected size. Shown is a polaroid image of an ethidium-stained, 1% agarose gel. *B*, immunoblot analysis of autocrine constituents in TRAMP C1 and TRAMP C2 was performed by immunoblotting with anti-EGFR (*top*, 7.5% protein gel) as well as anti-pro-TGFα (*bottom*, 15% protein gel) antibodies.

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signaling. Cells were then treated with 10 nM EGF, and the breast cell lines cell lysates were immunoprecipitated with anti-EGFR antibody and immunoblotted with antiphosphotyrosine, whereas cell lysates from the TRAMP cell lines were directly size-fractionated by SDS-PAGE. EGFR was markedly phosphorylated due to EGF stimulation in the cell lines with up-regulated EGFR (Fig. 2). These findings are consistent with previously published reports (40, 41) and serve to demonstrate that the lines we are investigating behave as reported.

In the breast cell line, MDA-361, which presents increased levels of the EGFR relative ErbB2, ErbB2 was found to be constitutively phosphorylated ErbB2 independent of the presence of heregulin or EGF (Fig. 2). MDA-361 cells also contain low levels of EGFR that are phosphorylated on EGF stimulation (data not shown). The slight decrease in phosphorylation of ErbB2 in MDA-361 cells upon ligand stimulation, which is a previously observed event (42, 43), is postulated to be due to ErbB2 redistribution in the plasma membrane, as well as increased degradation upon transphosphorylation or complexing with other ErbB-family members.

A central postulate of this study is that PLCγ-mediated signaling is operational in diverse tumor cell lines. Because the TRAMP model was only recently derived and characterized, we examined the responsiveness of TRAMP C1 and TRAMP C2 cells to EGF (10 nM) stimulation. Both TRAMP cell lines responded with a marked phosphorylation of PLCγ in response to EGF stimulation under conditions that minimize autocrine signaling (Fig. 3*A*). To demonstrate that this phosphorylation coincided with increased PLC

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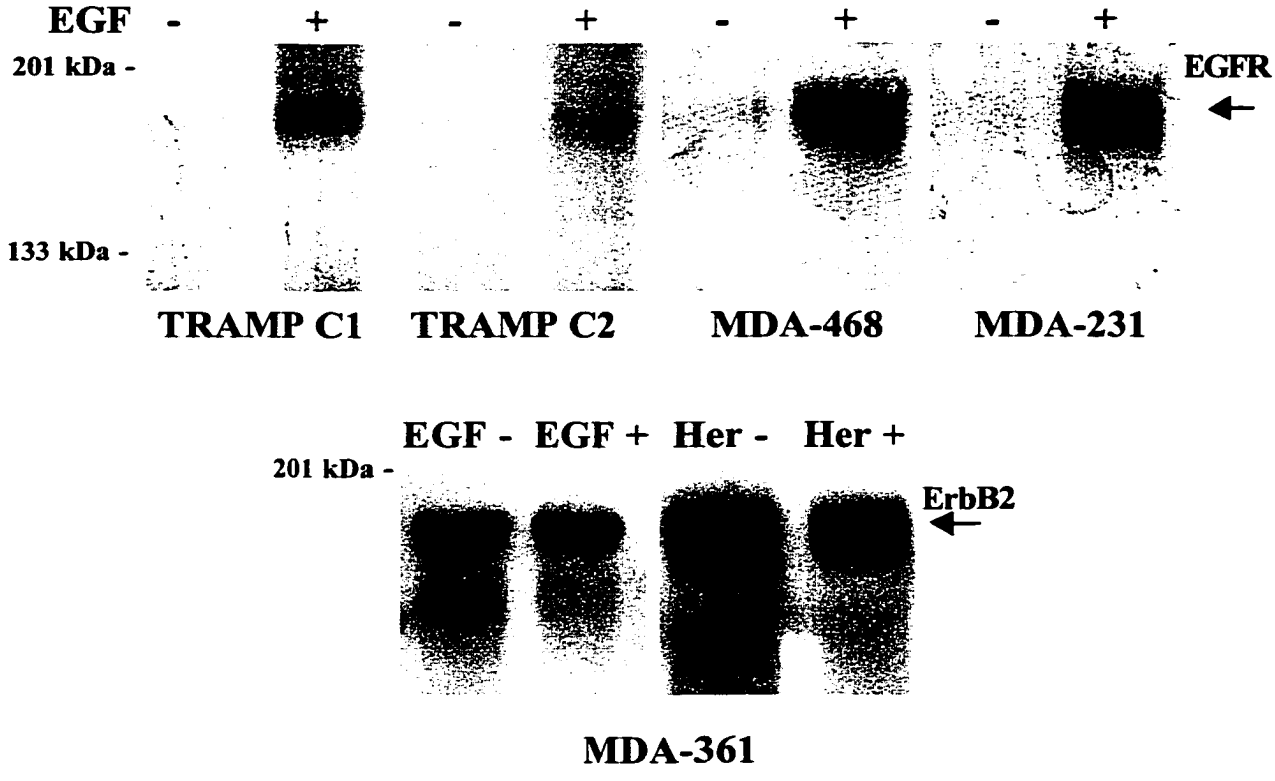

Fig. 2 Phosphorylation of EGFR and ErbB2 in target cells. EGF stimulates EGFR phosphorylation in TRAMP C1, TRAMP C2, MDA-468, and MDA-231 cells. For TRAMP cells, lysates of cells treated with 10 nM EGF or untreated were size-fractionated by SDS-PAGE and then immunoblotted with antiphosphotyrosine antibody (Transduction Laboratories; catalog #P11120). For other cell types, lysates of cells either treated with 10 nM EGF or untreated were immunoprecipitated with anti-EGFR antibody (Oncogene Science; catalog #GR01) and immunoblotted with antiphosphotyrosine antibody. *Bottom*, lysates of MDA-361 cells treated with 10 nM EGF or 50 ng/ml heregulin or untreated were immunoprecipitated with anti-ErbB2 antibody (Calbiochem; catalog #OP39) and immunoblotted with antiphosphotyrosine antibody. Immunoprecipitation using anti-EGFR performed on MDA-361 demonstrated negligible EGFR activity in response to EGF or heregulin (data not shown). Shown are representative immunoblots of at least two experiments each.

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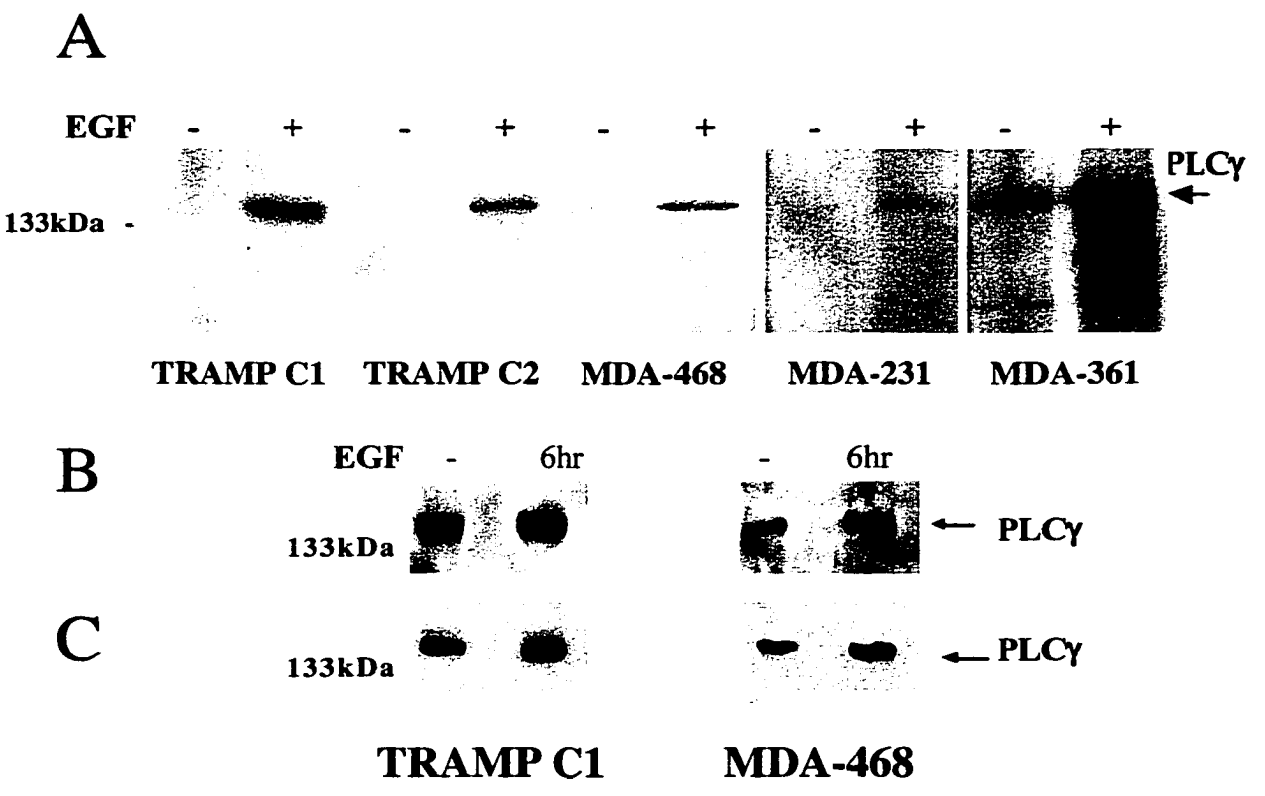

Fig. 3 EGF-induced phosphorylation of PLCγ in TRAMP, MDA-468, MDA-231, and the ErbB2-overexpressing MDA-361 cells. *A*, autocrine minimizing conditions demonstrate inducible PLCγ phosphorylation: all cells were grown to subconfluence and serum-starved for 24 h. For TRAMP cells, lysates of cells treated with 10 nM of EGF or untreated were immunoprecipitated with anti-PLCγ antibody (Upstate Biotechnology; catalog #05-163) and immunoblotted with antiphosphotyrosine antibody. For all other lines, cells were immunoprecipitated with antiphosphotyrosine antibodies and then immunoblotted with anti-PLCγ antibody. In MDA-361, PLCγ was constitutively phosphorylated as expected due to its overexpression of ErbB2. Shown are representative immunoblots of at least two experiments each. *B* and *C*, constitutive signaling from PLCγ in the absence of exogenous ligand. TRAMP C1 and MDA-468 cells exhibit baseline PLCγ phosphorylation after prolonged EGF exposure (6 h). Cells (two plates per lane) were incubated in complete media for 48 h at full confluence (*B*) and were also incubated for 24 h with serum-free media after remaining at confluence for 48 h (*C*), then treated with EGF for 6 h or left untreated. Lysates were immunoprecipitated with antiphosphotyrosine antibody and immunoblotted with anti PLCγ. Shown is a representative of at least three experiments.

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activity, the PLC inhibitor U73122 significantly reduced EGF-induced IP production in TRAMP C1 cells by 44% (data not shown).

To confirm that PLCγ is activated on ligand stimulation in all cell types, we also immunoprecipitated lysates of MDA-468 and MDA-231 cells treated or untreated with 10 nM EGF under autocrine signaling-limiting conditions with antiphosphotyrosine and blotted with anti-PLCγ antibody. These determinations, some of which confirm previously reported results (40, 41), serve as a necessary positive control for experiments to follow. Acute EGF treatment causes an increase in phosphorylation of PLCγ in these cells (Fig. 3*A*). PLC activity assays also were performed on MDA-468 cells. EGF treatment increased IP production significantly (1.8-fold), and pretreatment with U73122 decreased IP production by up to 76% (data not shown).

Increased PLCγ phosphorylation also was observed after EGF stimulation in the ErbB2-overexpressing MDA-361 cells (Fig. 3*A*). MDA-361 cells exhibited constitutively active PLCγ, which is expected due to its overexpression of constitutively active ErbB2. PLCγ phosphorylation increased with EGF treatment, presumably due to low EGFR levels in these cells that cross-phosphorylate ErbB2. Interestingly, previous studies indicate modulation of downstream phosphorylation by cytoplasmic (non extracellular domain-containing) EGFR (and possibly ErbB2) (44, 45). The significance of these results is simply that there is an inducible PLCγ pool in these cells.

PLCγ Signaling is Active In the Absence of Exogenously Added EGFR Ligand. The above manipulations demonstrated that the motility-associated PLCγ signaling can be induced in these cells lines. However, a central point of our model posits activa-

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tion of this motility pathway be dysregulated autocrine signaling in the case of up-regulated EGFR or constitutive signaling in the case of overexpressed ErbB2. As such, we tested whether PLCγ signaling was operative under autocrine signal-permitting conditions in the four EGFR-expressing cell lines. To address this question, cells plated under autocrine-promoting conditions were compared to those under autocrine-limiting conditions. Two of the cell lines (TRAMP C1 and MDA-468) were challenged for 6 h by EGF (Fig. 3, *B* and *C*). This latter condition was chosen to both represent the time period at which EGF-induced motility is maximal after an initial lag phase (46) and to allow for cellular adaptation with regulated down-regulation that would be operational during autocrine signaling. In this analysis, cells under autocrine-promoting conditions presented enhanced PLCγ phosphorylation comparable to the same cells under autocrine-restricting conditions or after extended EGFR signaling. Further support for constitutive activation of PLCγ signaling was noted by decreases in IP production in the presence of U73122 in TRAMP C1 (down to 57-66% in cells treated with or without EGF in the presence of U73122, when compared with cells treated with or without EGF in the absence of U73122).

Inhibition of PLC Signaling Reduces Cell Invasion *in Vitro*. Our previous studies using DU-145 prostate cancer cells implicated the EGFR-stimulated PLCγ motility pathway in tumor cell invasion (13, 14). We hypothesized that this role of the PLCγ is not unique to DU-145 cells. We predicted that this is especially true in breast and prostate that exhibit a high incidence of EGFR overexpression when transformed and, consequently, higher invasion rates and poorer prognoses. To this end, we assessed the *in vitro* invasiveness of the TRAMP C1, TRAMP C2, MDA-468, and MDA-231 cell lines. Cells were

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serum-starved and labeled with tritiated thymidine for 24 h, after which they were plated onto EGFR ligand-rich Matrigel-coated invasion chambers and allowed to invade for 72 h in the presence or absence of a pan-PLC inhibitor, U73122 (47, 48). The upper chamber contained media with 1% BSA for the first 24 h and was replaced with serum free media for the remaining 48 h, whereas the bottom (collecting) chamber contained media with 10% FCS throughout. In both prostate and breast cell types, those treated with U73122 invaded to a significantly lesser extent than nontreated cells (Fig. 4). Invasiveness of TRAMP C1 and TRAMP C2 cells treated with U73122 were reduced by 47% and 48%, respectively, compared to their nontreated counterparts, and invasiveness of MDA-468 and MDA-231 cells were reduced by 29% and 36%, respectively. These reductions in PLCγ motility pathway-modulated invasiveness compare favorably with those seen in DU-145 cells treated similarly; importantly, this was reflected *in vivo* by near complete abrogation of invasion (13-15). There were no observed differences in cell morphology or adherence to Matrigel at the concentrations of U73122 used when compared to nontreated cells. The reduced invasion of these cells as a result of PLCγ inhibition with U73122 emphasizes the important role of growth factor-induced PLCγ motility pathway in cell invasion, as shown using EGFR-expressing cells. Furthermore, the MDA-361 cells that overexpress a constitutively active ErbB2 likewise demonstrated reduced invasion when treated with U73122 (52%).

The observed inhibition of cell invasiveness by U73122 might be due to nonspecific drug toxicity. To test for such toxicity by examining a PLCγ-independent response, mitogenesis (11, 49), cells were grown on Matrigel-coated 24-well plates and treated with U73122 concentrations used during the invasion assays for each cell type. The cells were under identical conditions as in invasion assays (except that tritiated thymidine was added 8

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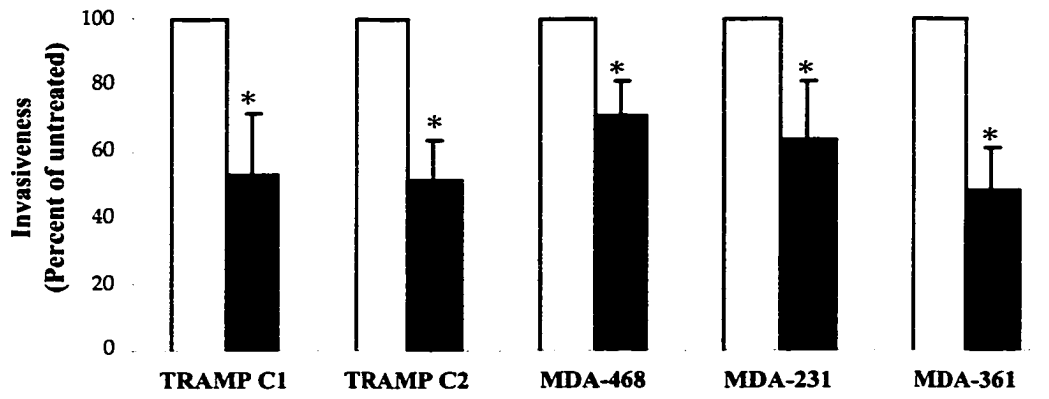

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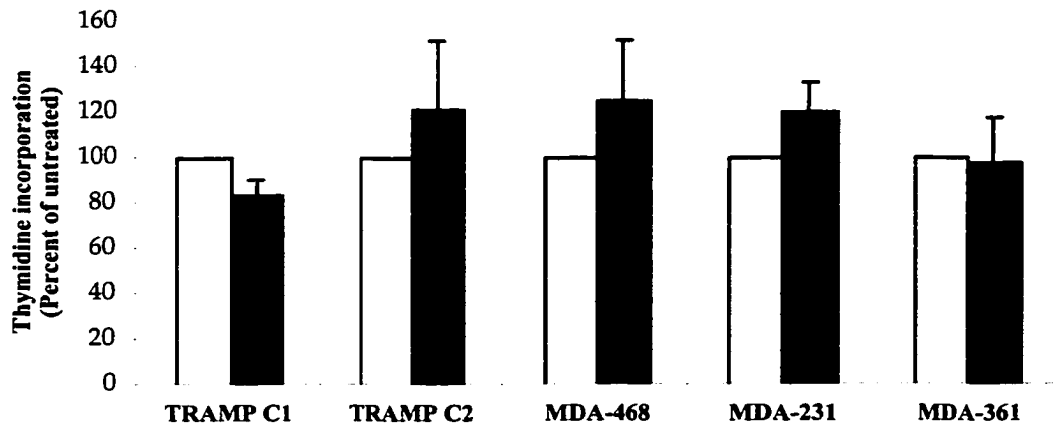

Fig. 4 Effect of U73122 on cell invasiveness through Matrigel. TRAMP C1, TRAMP C2, MDA-468, MDA-231, and MDA-361 cells were evaluated in their ability to transmigrate through a layer of Matrigel across a gradient for a 72-h time period (see "Materials and Methods"). Experiments were performed using modified Boyden chambers in either 6-well plate or 24-well plate configurations. Cells were plated in the following numbers: MDA-468, 200,000 cells/24-well plate; all others,100,000 cells of each type/24-well plate. All breast cells were treated with 3 μM U73122, and TRAMP cells were treated with 5 μM. *A*, significant inhibition of invasiveness by the PLC inhibitor U73122. *B*, no significant toxicity of U73122 as demonstrated by no effect on thymidine incorporation when the cells are grown Matrigel. Shown are mean ± SE of at least two experiments for each cell line, each performed in triplicate. ■ U73122-treated cells; □, controls; *, $P < 0.05$ comparing U73122-treated *versus* untreated.

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h before harvesting) for 72 h, after which incorporated thymidine counts were obtained. The cells treated with U73122 did not show any reduced thymidine incorporation when compared to nontreated cells (Fig. 4). Mitogenesis assays were also performed on cells in regular non-Matrigel coated plates and showed similar results of not affecting cell proliferation (data not shown). These results demonstrate that U73122 does not affect invasion by hindering cell mitogenesis or proliferative functions.

A Dominant Negative PLCγ Fragment Reduces Invasion In MDA-468 Cells. To further explore the role of the PLCγ motility pathway in tumor cell invasion, MDA-468 cells were engineered to express the dominant negative PLCγ fragment, PLCz. Cells were transfected with a constitutively transcribed PLCz (pXf PLCz) plasmid or a similar vector containing an irrelevant peptide (pXfcontrol). We used pooled clones to avoid cell-cell microheterogeneity. These cells were subjected to PLC activity assays as described above, and cells expressing PLCz exhibited a reduced IP yield compared with untransfected cells (Fig. 5), whereas cells expressing pXfcontrol did not. MDA-468 cells expressing PLCz were assayed for Matrigel invasion. Cells expressing PLCz showed reduced invasion (by ~50%) on the order of that noted in the presence of U73122.

Inhibition of EGFR Kinase Activity Reduces TRAMP C1 and MDA-468 Cell Invasion. The Matrigel invasion experiments were performed in the presence of EGFR ligands derived from cell autocrine signaling and present in Matrigel. Autocrine activation of PLCγ signaling was demonstrated by continuous phosphorylation of PLCγ despite the absence of exogenous EGF (Figs. 3*B* and 4*A*). This suggested that the PLCγ-

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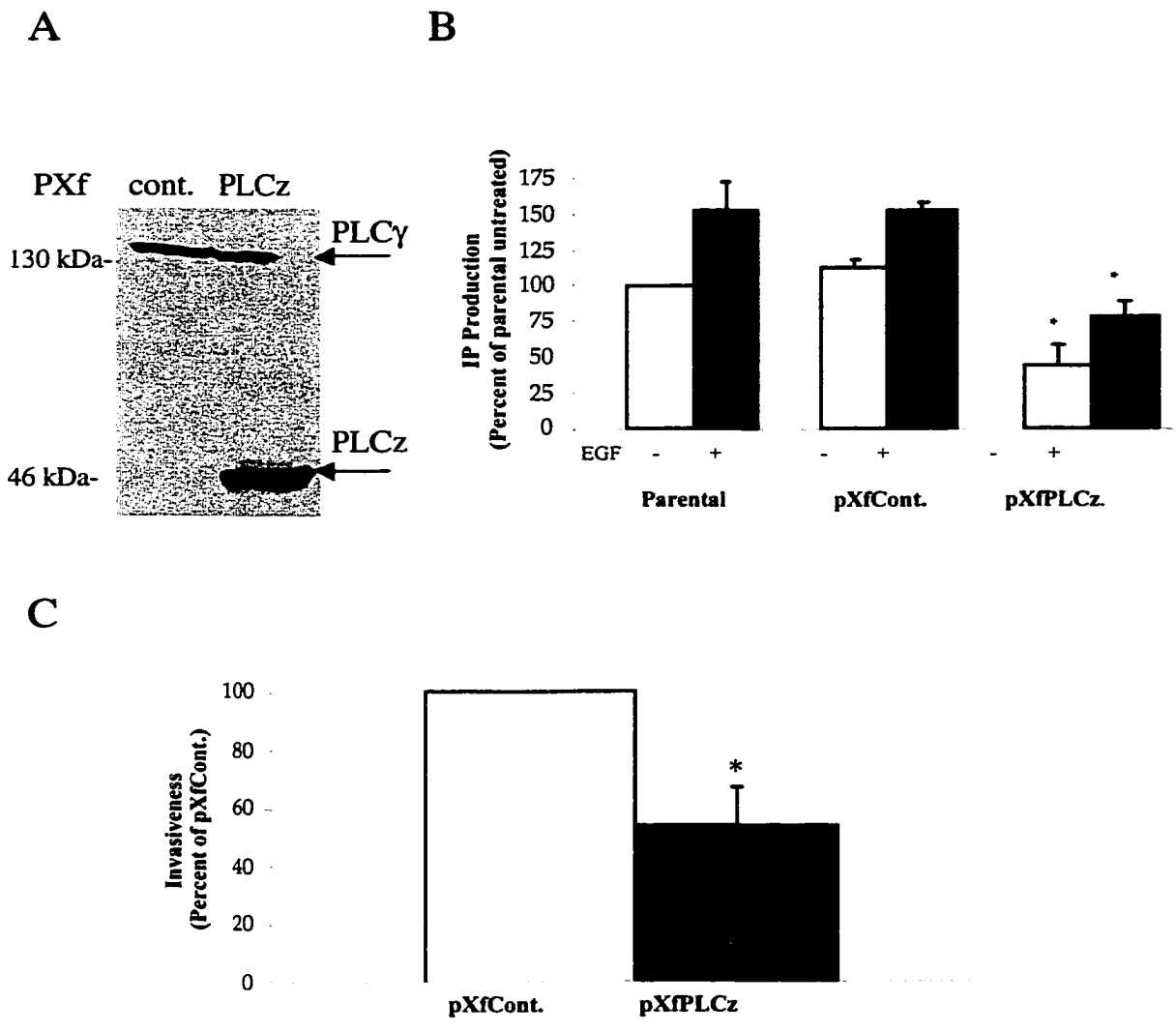

Fig. 5 Expression of PLCz in MDA-468 cells (*A*) and effects on PLC activity (*B*) and invasiveness (*C*). *A*, the dominant-negative PLCγ fragment PLCz was expressed stably in MDA-468 cells by lipid-mediated transfection of constitutively active pXfPLCz or pXfcontrol (as a negative control) and subsequent selection of a population of transfectants. The level of PLCz (~51 kDa) attained was in excess to that of endogenous PLCγ (140 kDa) as assessed by immunoblotting of cell lysates separated on 7.5% SDS-PAGE. *B*, parental MDA-468 cells, as well as MDA-468 pXf cells, were treated with 10 nM EGF for 30 min in the presence of 10 mM LiCl and then subjected to PLC activity assays to measure IP production, as discussed in "Materials and Methods." *C*, MDA-468 PLCz cells were subjected to invasion assays, as described previously, and compared to MDA-468 cells transfected with pXfcontrol. ■, MDA-468 cells expressing PLCz; □, MDA-468 cells expressing pXfcontrol (baseline). Shown are mean ± SE of at least two experiments (for IP production) or four experiments (for invasion), each performed in triplicate. Invasion of pXfcontrol cells was similar to parental cells in the two direct comparisons performed. *, $P < 0.05$ compared with the parental or pXfcontrol cells under similar treatment.

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dependent aspect of invasiveness is secondary to EGFR signaling based on the presence of a potential autocrine stimulatory loop. However, to determine whether EGFR signaling was actually involved in invasion, we inhibited EGFR signaling and thereby the presumed downstream activation of PLCγ using the EGFR kinase-specific inhibitor PD153035 (50). TRAMP C1 and MDA-468 cells were treated with PD153035, and invasion assays were performed as described above. Invasion wells containing 100,000 TRAMP C1 cells or 200,000 MDA-468 cells each were treated with PD153035 (500 nM, determined empirically as the concentration needed to inhibit EGFR-induced phosphorylation) and the invaded cells were quantitated (Fig. 6). Invasiveness of TRAMP cells treated with PD153035 was reduced by 32% compared with their nontreated counterparts, whereas invasion of MDA-468 cells was reduced to 45%. These reductions in invasiveness are similar to those seen with the pharmacological inhibitor of PLC. One concern is that inhibition of EGFR signaling reduces cell proliferation and this may be seen as fewer cells transmigrating the Matrigel barrier; however, as the TRAMP C1 cells were metabolically labeled and enumerated, this assay is independent of cell proliferation. Furthermore, there were no observed differences in cell morphology, adherence to Matrigel, or cell survival at the concentrations of PD153035 used when compared to nontreated cells.

DISCUSSION

Up-regulated signaling from EGFR or erbB2 is strongly correlated with tumor invasion and metastasis, although the responsible intracellular events remain undefined. Taking into account the sequence of events that are involved in tumorigenesis and progression, our studies have focused on cell motility under the hypothesis that this event modu

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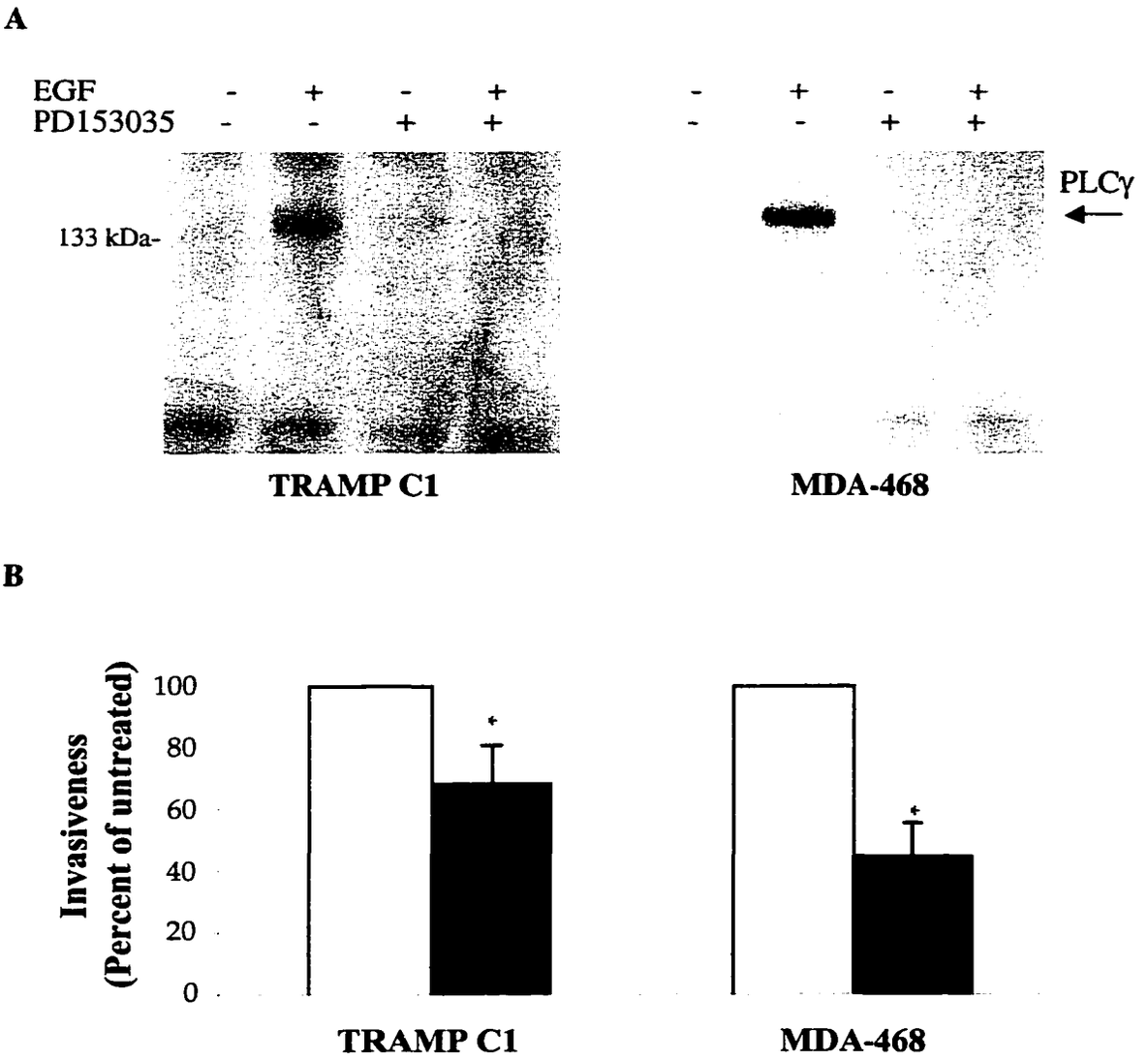

Fig. 6 Effect of PD153035, an EGFR kinase inhibitor, on cell invasiveness through Matrigel. *A*, PLCγ phosphorylation in TRAMP C1 and MDA-468 cells is inhibited by PD153035. Cells were starved in media containing 1% dialyzed FCS for 24 h, after which plates were treated with 500 nM of PD153035 for 25 min at 37°C, followed by 10 nM EGF for an additional 5 min. Lysates were immunoprecipitated with anti PLCγ antibody and then probed with PY20 antiphosphotyrosine antibody. *B*, TRAMP C1 and MDA-468 cells were evaluated for transmigration through a layer of Matrigel, as described. Cells were plated in the following numbers: TRAMP cells, 100,000 cells/24-well plate; and MDA-468, 200,000 cells/24-well plate. ■, treated cells; □, controls; *, $P < 0.05$ compared to untreated cells (mean ± SE, n≥3)

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lates tumor invasion and metastasis (51, 52). In this study, we demonstrate that disrupting the motility-associated PLCγ-mediated signaling pathway inhibits *in vitro* invasion in multiple cell types.

This signaling contributing to invasion was first described by our laboratory in the human prostate cancer cell line DU-145 (13-15). These prior studies were vital for establishing proof of the concept that EGFR-enhanced migration can be a rate-limiting step in theinvasion of a tumor. However, questions remained as to whether this critical role of the motility pathway in invasion is unique to DU-145 cells only, whether it is prostate cancer-specific, or whether it is a more universal concept that holds in a variety of different tumor types. The studies presented here investigated the role of the EGFR-induced PLCγ-mediated motility pathway in cell invasion in a different, *de novo*-occurring mouse prostate carcinoma (the TRAMP model) and also extended this concept to other tumors of the steroid-responsive tissue, namely breast (MDA-468 and MDA-231). Lastly, because PLCγ is a point of convergent signaling from multiple growth factor receptors (21, 22), we determined whether PLCγ-mediated signaling was also required for cell invasiveness in a breast cancer line, MDA-361, overexpressing the closely-related erbB2 receptor. In these experiments, ErbB2 phosphorylation levels decreased slightly upon stimulation with EGF. This is a previously observed occurrence that is due to EGF modulating increased internalization and down-regulation rates of EGFR-ErbB2 heteromer aggregates (42, 43).

Our hypothesis postulates that EGFR-induced cell migration is a major regulatory step in tumor progression. Disruption of this pathway, through specific inhibition of PLCγ, should, therefore, reduce cell invasiveness. We treated cells with U73122, a pharmacological agent that specifically inhibits PLC and, as such, inhibits cell motility but not mitogene-

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sis both in fibroblasts (11, 49) and prostate epithelial cells (13). Ancillary studies showed that cells treated with U73122 demonstrated reduced IP production on EGF stimulation in a drug dose-dependent manner (data not shown), indicating that U73122 inhibited PLCγ in cells regardless of tissue of origin. In our earlier studies in fibroblasts (11), we found that PLCγ signaling levels controlled the extent of migration and that even partial inhibition of enzymatic activity was linearly related to extent of cell motility. Thus, if motility was rate-limiting for invasion, the relative decreases in IP production in the presence of U73122 should be reflected by similar decreases in Matrigel transmigration. It should be noted that although significant, inhibition is not complete. Cells usually exhibit some degree of base-line nonligand-induced motility, which likely contributes to some invasiveness in this *in vitro* assay. Interestingly, when examining invasiveness of DU-145 variants, we found that the cells expressing the nonmotility inducing c'973 EGFR presented 60% of the invasive-ness of parental cells *in vitro*, but were almost completely noninvasive *in vivo* (13, 15). Our more recent preliminary data suggest that PLCγ signaling is also important in invasiveness of cells from bladder carcinoma and glioblastomas, two other tumors in which up-regulated EGFR correlates with progression (53-55).

These studies strongly suggest that PLCγ-mediated signaling is a generalizable property for tumor cell invasiveness induced by growth factor receptors and not unique to DU-145 prostate carcinoma cells. The possibility that this decrease in invasion may be due to a toxic effect of U73122 was rendered unlikely due to the apparent nonsensitivity of cells to U73122 in terms of mitogenesis. U73122 is also unlikely to be affecting other signaling pathways because it is, by all accounts, targeted toward PLC and not even PLD or PLA_2 (47). However, to enhance specificity, we performed a similar set of analyses in which PLCγ

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signaling was abrogated by expression of an exogenously encoded dominant-negative fragment, PLCz (14, 35, 56). At the concentration and in the manner used, neither U73122 nor PLCz completely inhibit PLC activity (11), and, as such, we have not considered the difference in inhibition among the two methods as biologically significant. However, invasiveness was reduced to levels similar to or lower than those obtained in the earlier DU-145 studies utilizing either method of inhibition of PLC; these partial levels of *in vitro* inhibition corresponded to almost complete inhibiton of invasion *in vivo* (13-15). The results of both the PLC activity and Matrigel transmigration assays mirrored those obtained using U73122, which strongly supports a specific role for the PLCγ-mediated motility pathway in tumor invasion.

It may be noted that the various tumor cell lines transmigrated the Matrigel barrier in the absence of exogenously added EGFR ligands. The addition of EGF was deemed unnecessary for two reasons. First, prostate and breast carcinoma cell lines often present autocrine stimulatory loops involving the EGFR and its ligands (2-5). The TRAMP cells present both EGFR and EGF and TGFα (Fig. 1) and are dependent, at least in part, on EGFR signaling for mitogenesis. The EGFR-expressing breast cell lines MDA-468 and MDA-231 cells have been reported also to possess an autocrine stimulatory loop and, thus, present up-regulated EGFR signaling (57, 58). Our experimental data also point to autocrine activation of EGFR signaling, as we have determined that the EGFR-specific inhibitor PD153035 limits TRAMP cell line mitogenesis even in the absence of added exogenous ligand or matrix (data not shown). We do not imply that invasion and motility are signaled solely via PLCγ; other intermediary signaling pathways are involved. However,

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our findings simply show that invasiveness seen in these cells occurs secondary to PLCγ- and EGFR-mediated signaling.

It was difficult to demonstrate autocrine signaling at the level of EGFR autophosphorylation as ligand-induced down-regulation under autocrine-promoting conditions resulted in constitutively low levels of EGFR similar to 6 h treatment with EGF (data not shown). This result was expected from previous detailed analyses of EGFR trafficking in response to autocrine stimulation in which ligand is in excess (59, 60). Thus, we inferred the autocrine loop by the expression of both ligand and receptor and the effects of PD153035 on downstream signaling. This autocrine signaling is likely sufficient in itself because we found earlier that DU-145 cells transmigrated a human matrix barrier in an EGFR-dependent manner, although the Amgel does not contain detectable levels of EGFR ligands (15, 61). Second, Matrigel contains high levels of EGFR ligands (62). While the presence of copious growth factors, including PDGFR and TGFβR ligands in Matrigel confounds *in vitro* analyses, it may be physiologically representative of the organismal situation in which stromal cells produce TGFα and other EGFR ligands (notably amphiregulin and heparin-binding EGF) that are often present in extracellular matrices (62, 63). Thus, it is likely that in the presence of Matrigel both the matrix-associated ligands and the autocrine signaling sufficiently activate the overexpressed EGFR. On the other hand, it is possible that we disrupted a signaling pathway that is not actually up-regulated in these tumor cells, but rather the basal signaling of which is required for active cell motility. As such, PLCγ signaling would be considered as permissive for invasiveness rather than as a consequence of up-regulated EGFR or ErbB2 signaling. We favor the former possibility because disruption of EGFR signaling with the EGFR kinase-specific inhibitor PD153035

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reduced invasiveness of TRAMP C1 and MDA-468 cells similarly to PLCγ abrogation. In either case, whether PLCγ signaling is actively regulated or simply permissive, this signaling pathway would still be a target for rationale therapeutic intervention to limit tumor invasion.

It is important to state that enhanced cell motility is not the only rate-limiting step or cell behavior with regards to tumor progression and invasiveness. Tumor progression can be limited by blocking other required cell events, such as cell proliferation and production of proteolytic enzymes (64, 65). In many cases, it is not clear whether these cell events are promoted at the transition to invasiveness or are present in a permissive manner at this stage from earlier cell alterations (66). It also is likely that other signaling pathways, such as those involving PI-3 kinase and mitogen-activated protein kinase (22, 67, 68), are important for signaling motility, and abrogation of those would similarly limit invasiveness. We did not pursue these because PI-3 kinase phosphorylation was not induced by EGF in our cells (data not shown) and mitogen-activated protein kinase was considered too broad a target, which would also interrupt cell proliferation (68, 69). Although there is evidence that the upregulated EGFR and ErbB2 receptors signal increase proliferation (70) and production of select proteases (71), future studies are required to determine if these particular events are linked to receptor up-regulation and its correlation to tumor invasion.

In summary, these data strongly suggest that the role of the EGFR- PLCγ cell motility pathway in tumor invasion encompasses a number of different cell types and suggest that the requirement of cell migration for invasion may be a universal concept. Ultimately, whether this is actually the case will require studying the invasiveness of these cells, along with inhibiting the PLCγ motility pathway in an *in vivo* environment. Such experiments would require suitable cell lines, such as MDA-231, that are relatively invasive *in vivo* com-

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pared to MDA-468 (72). Also of significance is that these results suggest that ErbB2 can subsume the role of EGFR in so far as activating PLCγ signaling. The results presented here also provide for a possible therapeutic approach in which specific targeting of the motility-inducing mechanisms may provide a treatment against tumor progression.

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A ROLE FOR PHOSPHOLIPASE C-γ-MEDIATED SIGNALING IN TUMOR CELL INVASION: SUPPLEMENT

The findings described in the previous manuscript are further supported by the following study in which two transgenic mouse lines were utilized. The aim of this study was to explore the concept of PLCγ being rate limiting for tumor invasion *in vivo*. In order to perform this study, we required an animal model in which metastatic tumors arise *in situ* (to simulate a patient model). To this end, we used transgenic mice that constitutively express polyoma virus middle-T cDNA driven by a mouse mammary tumor virus-long terminal repeat (MMTV-LTR) promoter. These mice produced highly invasive mammary tumors that metastasize to the lungs with 100% frequency (Fig. 1*A*). We also required an inducible PLCz expressing system in order to control PLCz gene expression. This was accomplished by using transgenic mice that express tetracycline-inducible PLCz cDNA, which is localized only to the breast and prostate via a tissue-specific promoter [C3(1) prostatein] (Fig. 1*B*). Double transgenic mice were bred from these two lines.

Mice were treated with 2 mg/ml of doxycyclin (a tetracycline analog) in their water starting at 3 months of age, with treatment continuing until 5 months of age when they were sacrificed. During this time, diameters of primary and secondary tumors that surfaced were measured twice weekly. The periods of treatment were determined to be optimal by previous testing prior to onset of experiments. Upon death, the lungs of the mice were removed, fixed in 10% formalin, paraffin-embedded, and stained with

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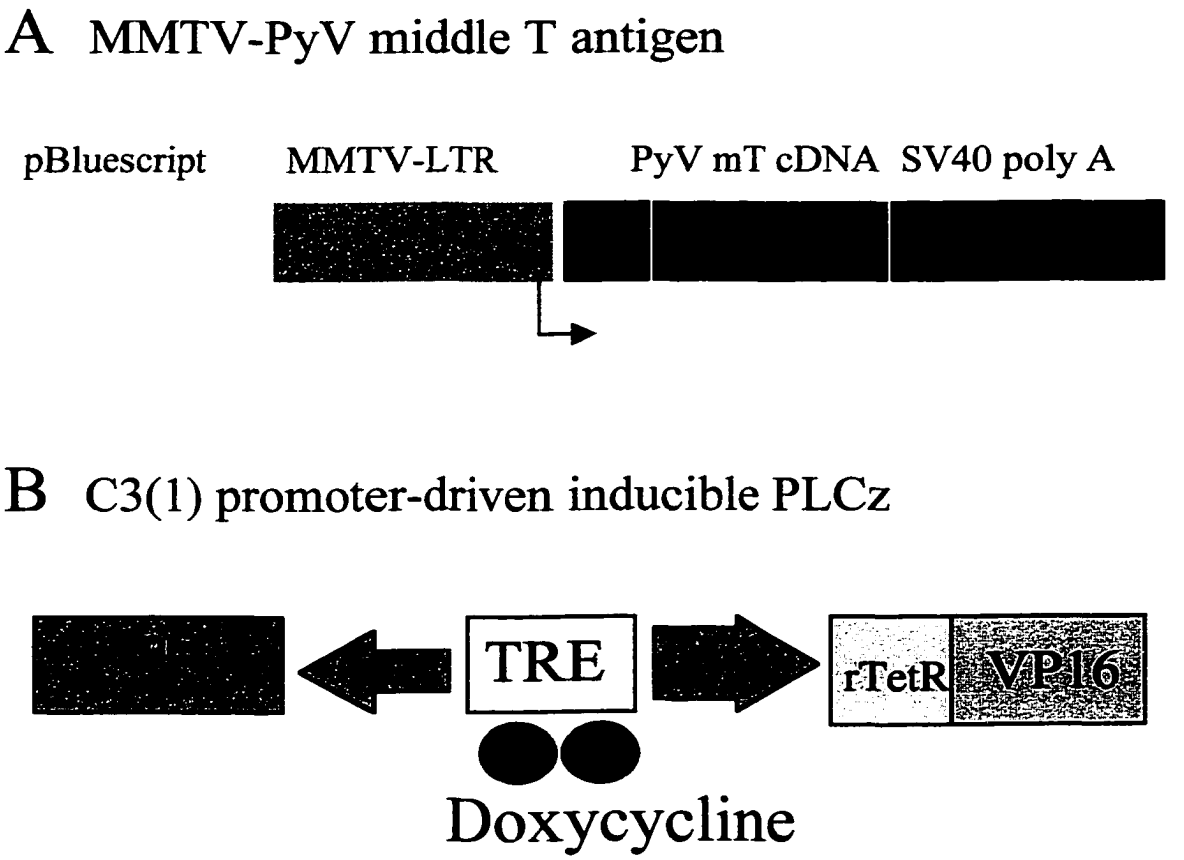

Fig. 1 Schematics of the transgenic genes constructed and expressed in mice. *A*, the polyoma middle-T antigen is constitutively expressed via the MMTV-LTR promoter. *B*, the C3(1) promoter, which is only active in breast and prostate tissues, drives the transcription of the rTetR/VP16 protein, which only in the presence of doxycycline binds to the tet-response-element to drive expression of PLCz.

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hematoxylin and eosin (H&E). Sizes of metastases were measured by microscopy analysis of histology slides.

Treating the double transgenic animals with doxycycline reduced sizes and incidences of metastases to the lung (Figs. 2 and 3). These results constitute a major confirmatory finding of the concepts introduced in the first manuscript, which in its entirety provides a cornerstone for the assertion that growth factor-mediated cell migration is a rate-limiting step in tumor cell invasion.

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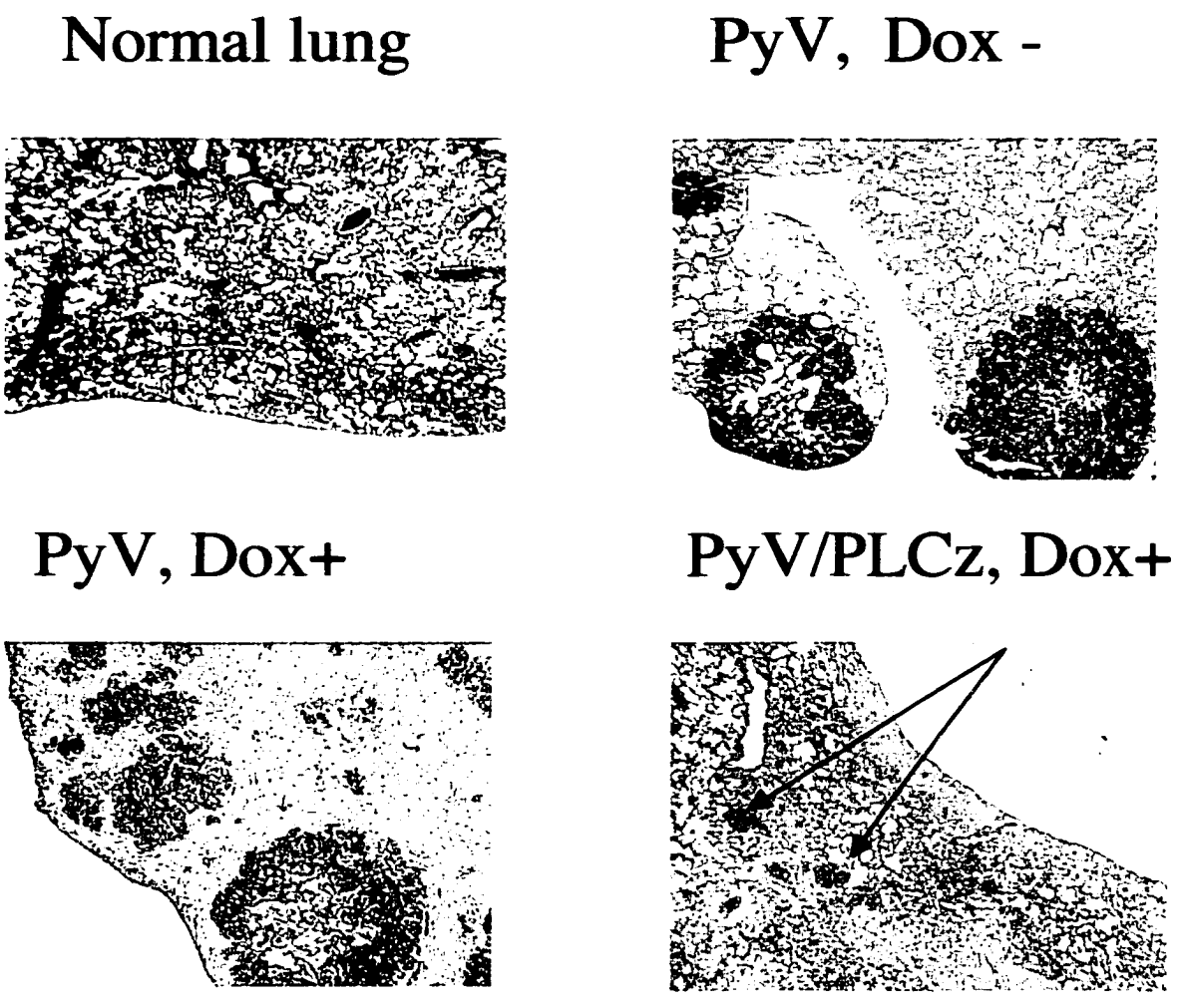

Fig. 2 Lung metastases of mammary tumors in transgenic mice expressing the PLCz dominant negative. Transgenic mice carrying polyoma middle-T cDNA driven by an MMTV-LTR promoter were mated with mice expressing tetracycline-inducible PLCz cDNA, which was localized only to the breast and prostate via a tissue-specific promoter [C3(1) prostatein] to establish a population of double-transgenic mice carrying both constructs as verified by PCR. Mice were treated with 2 mg/ml doxycycline, as placed in their water, for 60 days after which lungs were removed, fixed in 10% formalin, paraffin-embedded, and stained with H&E for histological evaluation. Control mice treated with doxycycline expressed only the first construct (polyoma middle-T).

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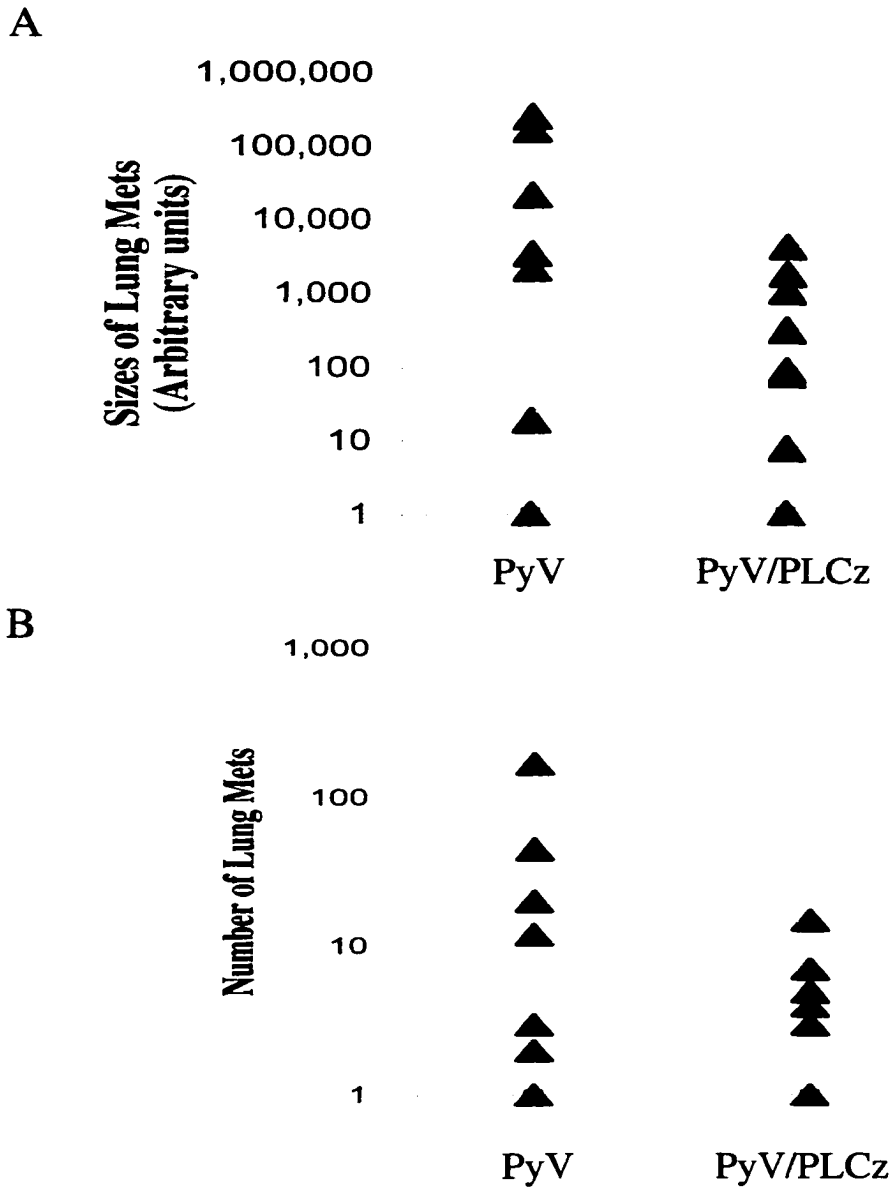

Fig. 3 Summary of metastases of mammary tumors in transgenic mice to the lungs. *A*, diameter of each metastasis were measured using microscopy, from which tumor size was calculated as a volume of a sphere. Shown are the ranges of sizes found on each lung. *B*, number of metastases were counted on each histological slide for each lung, n = 10 for each of PyV and PyV/PLCz.

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MOTILITY IS RATE-LIMITING FOR INVASION OF BLADDER CARCINOMA CELL LINES

by

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ABSTRACT

Induced migration of tumor cells is generally considered to be a critical step in cancer progression to the invasive and metastatic stage. This conclusion is based on many studies in which individual specific molecular targets are abrogated, resulting in concomitantly decreased invasion and diminished motility. The implicit caveat of such studies is that other, unknown, signaling pathways and biophysical events are actually the operative rate-limiting steps and not motility per se. Thus, to examine the hypothesis that motility is rate-limiting for invasion, disparate motility processes need be blocked with concordant effects on tumor invasion. Recently, we and others have described two signaling pathways that are critical to growth factor-induced motility but not mitogenesis. The key molecular switches are phospholipase C (PLC)γ and calpain for cytoskeletal reorganization and rear detachment, respectively. We examined this hypothesis in a highly invasive tumor, bladder carcinoma. Three different human tumor cell lines, 253J-B-V, UMUC-2, and T-24, were tested for invasiveness *in vitro* by transmigration of a Matrigel barrier. Inhibiting PLCγ with the pharmacologic agent U73122 or the molecular dominant-negative PLCz construct reduced both invasiveness and motility. The same was noted when calpain was blocked using calpain inhibitor I (ALLN). These results demonstrate that the interventional target for limiting invasion is not necessarily an individual pathway but rather cell migration per se.

INTRODUCTION

A defining stage of a growing tumor is the acquisition of ability to invade into surrounding tissue (1). While localized tumors are usually curable by excision, invasive tumors pose treatment challenges and often lead inextricably to death. Thus, work has

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focused on the molecular changes that provide the invasive capabilities. Invasion into adjacent tissue requires the cell to recognize the barrier matrix, breach the matrix, and grow in the ectopic locale. Dysregulation of induced cell motility has been postulated to be operative in the transmigration of the barrier matrix (2-4). However, as this model has been based upon disruption of specific molecular actions, there runs the risk that the noted correlations between diminished motility and reduced invasion are due to secondary, unknown effects of molecular abrogation. As an example, we have found that inhibition of phospholipase C(PLC)-γ signaling in prostate carcinoma cells affects the expression of a small fraction of genes, some of which also contribute to cancer progress (5; Kassis *et. al.*, unpublished data). Herein, we tested the hypothesis that cell motility is critical for invasion by targeting two independent modulators of diverse biophysical processes of cell motility in an invasive tumor type, bladder carcinoma.

Therapeutic intervention necessitates the identification of specific molecules even if an integrated biological response is the true target. Growth factor-induced cell motility occurs via at least two distinct signaling switches that modulate separate biophysical responses. The first is PLCγ, being directly activated by receptors with intrinsic tyrosine kinase activity. This molecule is required for motility induced by the receptors for epidermal growth factor (EGF) (5), platalet-derived growth factor (6), and insulin-like growth factor-I (7), but seemingly not for haptokinetic and haptotactic migration signaled through integrin binding to substratum (8). PLCγ hydrolyzes phosphatidylinositol bisphosphate (PIP_2) with subsequent mobilization/activation of actin binding proteins such as gelsolin (9). This places PLCγ signaling as critical for cytoskeletal reorganization and membrane dissociation from the underlying infrastructure that enables lamellipod protrusion (10, 11). Inhibition of PLCγ does not prevent epidermal growth factor

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receptor (EGFR) mitogenic signaling, enabling this target to be a probe for motility-dependent events (12, 13). Abrogation of this signaling pathway prevents tumor invasion in both *in vitro* and *in vivo* models. Murine xenografts of human prostate carcinoma grew to large sizes but did not invade when PLCγ was inhibited either by a pharmacological agent, U73122, or a dominant-negative construct, PLCz (13, 14). Invasion of normal brain tissue by glioblastoma cells and EGF-, platelet-derived growth factor (PDGF)- or insulin-like growth factor-I (IGF-1)-induced motility, but not tumor cell growth, was similarly prevented by PLCγ inhibition (15). Breast carcinoma transmigration of a Matrigel barrier also depends on PLCγ signaling, whether driven by EGFR or ErbB2 (16). Further supporting the role of PLCγ signaling in tumor invasion, but confounding the simple interpretation that the only operative pathway is through epigenetic motility, is the finding that prostate tumor invasiveness appears to also require PLCγ-dependent transcriptional changes (17), though it is likely that some of these genes serve to reinforce the motility response (Kassis *et. al.*, unpublished data). Thus, while PLCγ can be specifically targeted to abrogate both growth factor-induced motility and tumor invasiveness, the direct, epigenetic causal nature of these two processes is still open to question.

The ubiquitous calpain isoforms represent a second molecular switch required for cell motility signaled both via growth factor receptors (18, 19) and integrins (20). Calpains are required for rear detachment on moderately to highly adhesive surfaces (21, 22). M-calpain (or calpain II) is activated by growth factors downstream of extracellular-signal regulated kinase (ERK)/mitogen-activated protein kinases (MAPKs) (18), whereas μ-calpain (or calpain I) appears to be activated by cytosolic calcium fluxes (20, 23). Despite the fact that the operative downstream target(s) of calpain have yet to be been

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identified, the evidence is clear that calpain proteolytic activity is necessary for uncoupling cells from the surface. Inhibiting calpain not only prevents growth factor-induced cell motility but also actually channels the contractile forces to matrix contraction (24). These findings place calpains as a central modulator of the biophysical process of deadhesion during cell motility. However, calpains are implicated in other cellular processes, including proliferation (25, 26), survival (27), and differentiation-related functions (28, 29), again raising the question of causality of limiting calpain to abrogate invasiveness.

The hypothesis that cell motility is rate limiting can be tested by abrogating specific activators of distinct biophysical processes and observing similar results. This is now possible due to the recent identification of two different molecular switches, PLCγ and calpain, that modulate distinct processes, protrusion and rear detachment, respectively. Bladder carcinoma is an attractive target for such an analysis. It is a highly prevalent cancer, afflicting around 54,000 patients annually and causing death in over 20% of these cases in the United States alone (30). Early invasion through the bladder muscle wall causes most of the morbidity and mortality, whereas superficial carcinomas are curable. To study the role of motility in invasion we chose the highly invasive and metastatic human bladder carcinoma subline 253J-B-V, which is a variant derived from the 253J parental line (31). For confirmation, we assessed the invasiveness of two other human prostate cell lines, T24 and UMUC-2 (32, 33). We found that, in the 253J-B-V cells, PLCγ is activated in an EGFR autocrine stimulatory loop. Inhibition of PLCγ clearly diminished Matrigel transmigration. While these results are significant in themselves and form the first report of invasion-related signals in bladder carcinoma, we also found that inhibition of calpain significantly limited invasion of these cells through Ma-

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trigel. These results, which limit invasion by disrupting two independent arms of growth factor-induced motility, strongly support the universal concept that motility per se is a rate limiting and vital component of tumor progression to the invasive state.

MATERIALS AND METHODS

Cell lines. The target human bladder carcinoma lines were 253J-B-V (31), T-24 (32), and UMUC-2 (33), of which the latter two were a kind gift from Dr. Robert Getzenberg (University of Pittsburgh, Pittsburgh, PA). 253J-B-V and T-24 cells were maintained in low-glucose DMEM containing 10% FCS and supplemented with L-glutamine (2 mM), penicillin/streptomycin (100 units/ml), nonessential amino acids (0.1 mM), HEPES buffer (25 mM), and sodium pyruvate (1 mM). UMUC-2 cells were maintained in RPMI-1640 media containing 10% FCS and supplemented with L-glutamine (2 mM), penicillin/streptomycin (100 units/ml), nonessential amino acids (0.1 mM), and sodium pyruvate (1 mM). All media and supplements were obtained from Life Technologies, Inc.

Expression of Dominant-Negative PLCγ Fragment PLCz. Introduction of exogenously-encoded PLCz was accomplished using electroporation. PLCz is a dominant-negative fragment of PLCγ and consists of the SH2 and SH3 domains and an inhibitory (I) domain (34). The constitutively expressed PLCz (pXf PLCz) was transcribed from an SV40 early promoter. Briefly, the DNA (~1 μg) was added to a suspension of 253J-B-V cells in optiMEM media and electro-pulsed (500 μF, 0.500 kV), after which the suspension was plated in complete media. Media was replaced after cells settled, and the cells were incubated for a further 48 h before use. Expression was verified by co-

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electroporation with Green Fluorescent Protein (GFP), which indicated ~50% expression rate. The 48 h incubation was established empirically as optimal time to highest GFP fraction and level of expression.

Immunoprecipitation/Immunoblotting. Protein expression and phosphorylation were determined as previously described. Cells were quiesced in media supplemented with 1% dialyzed FCS (dFCS) for 24 h, followed by stimulation with EGF (10 nM) for 5 min. Cells were washed with PBS and lysed in lysis buffer [10% glycerol, 1% Triton X-100, 100 nM NaCl, 20 mM HEPES (pH-7.4), and 1 mM sodium vanadate] for 1 h at 4°C. After clarification by microcentrifugation, the lysate was incubated for 1.5 h with a specific antibody-protein G agarose bead mixture that had been incubated overnight at 4°C. Primary antibodies included antihuman EGFR (Oncogene Science; catalog #GR01), anti-PLCγ antibody (Upstate Biotechnologies; catalog #05-163), and anti-phosphotyrosine antibody (Transduction Laboratories; catalog # P11120). The lysate-antibody-agarose bead mixture was then washed three times with lysis buffer prior to analysis by reducing SDS-PAGE and immunoblotting.

Migration Assays. Cell migration was determined by the *in vitro* wound healing method (35). Cells were grown to confluence in 6-well plates and then placed in media containing 1% dialyzed FCS for 24 h. The monolayer was then "wounded" with a rubber policeman. After wounding, cells were placed in 1% dFCS media containing the appropriate treatment. Using a microscope, the wound area was photographed initially and after 24 h. The distance that cells had traveled into the denuded area was determined using computer-driven image analyses, at three distinct points in the wound, and aver-

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aged. Cells were in quiescence media during the 24 h after wounding. For each wound, three individual measurements of the gap were taken. All experiments were performed in either duplicate or triplicate wells.

Calpain Activity Assays. Calpain activity in 253J-B-V cells due to EGF stimulation was determined using the Boc assay method. Cells were plated at 50% confluence on glass coverslips. The cells were then treated with CI-1 (ALLN) and incubated at 37°C for 1 h. T-butoxycarbonyl-Leu-Met-chloromethylaminocoumarin (Boc-LM-CMAC) (Molecular Probes) (0.5 μM) was added 40 min into CI-1 treatment, followed by10 nM EGF 15 min later; control cells were not treated with EGF. Boc-LM-CMAC is retained within the cells by conjugating with intracellular thiol groups. Cleavage of the substrate results in retention of the chloromethylami-nocoumarin portion of the molecule in the cell and results in increased fluorescence. The coverslips were then mounted on glass slides and observed for chloromethylaminocoumarin fluorescence using an Olympus fluorescent microscope (model BX40 with an Olympus M-NUA filter). Representative images of each slide were captured using a SPOT CCD camera.

Transmigration Assays. Cell invasiveness in *vitro* was determined by the ability to transmigrate a layer of extracellular matrix, Matrigel, in a modified Boyden chamber assay (36, 37). Matrigel invasion chamber plates were obtained from Becton Dickinson/Biocoat (catalog # 40480). For each individual cell line, cells were plated randomly and distributed among plates with different lot numbers, with each experiment performed in duplicate or triplicate. Due to variation among lot numbers due to Matrigel thickness and texture, each lot was individually validated with necessary control. Despite

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possible variances in EGFR ligand concentrations in Matrigel, these concentrations are saturating since even "reduced growth factor Matrigel" contains relatively high amounts of EGF (up 0.5 ng/ml compared to 0.5-1.3 ng/ml in regular Matrigel) (Becton Dickinson Labware 1997/98 catalog description, page 128). Cells were kept in serum-free media containing 1% BSA for the first 24 h and then replaced with serum-free media for a further 48 h (or further 24 h depending on cell type and treatment). Enumeration of the cells that invaded through the matrix was accomplished by removing uninvaded cells with a cotton swab, staining with crystal violet, and visually counting all cells on the bottom of the filter, as per routine procedures (36).

Mitogenesis Assays. Cells were plated into 12-well plates, allowed to grow to confluence, and then placed in serum-free media for 48 h. Following this, appropriate wells were treated with 10 nM EGF and/or appropriate concentrations of U73122 for a further 18 h. Five micro curies/ml [methyl-[^{3}H]] thymidine was added to each well, and incubation resumed for another 8 h. Wells were then washed with PBS followed by acid precipitation (5% TCA, 4°C for 30 min). The wells were washed with PBS and then treated with 0.2 N NaOH to solubilize the incorporated radiolabel. Scintillation counting quantitated the amount of incorporated thymidine. Each experiment was performed in duplicate.

Statistical Analyses. All analyses were performed as paired Student's *t* tests, with a level of significance assigned at < 0.05.

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RESULTS

PLCγ Signaling Pathway is Operative in Bladder Carcinoma Cells. Our model of tumor invasion invokes dysregulated signaling from growth factor receptors driving motility inappropriately to the situation (1, 2). One major pathway that controls this is that through the convergent molecular switch PLCγ. Thus, the initial question was whether this pathway was functional in bladder carcinomas. Three different human bladder carcinoma cell lines, 253J-B-V, T-24, and UMUC-2, were placed in quiescent media under conditions that minimized autocrine signaling (16) and challenged with EGF. This resulted in increased phosphorylation of PLCγ (Fig. 1). Additionally, it was noted that there was significant phosphorylation even in the absence of added EGF. Under the experimental conditions used, even robust basal PLCγ signaling is noted as only being at a fractional level of acute EGF-induced phosphorylation (16). As invasive bladder carcinomas present autocrine signaling through the EGF receptor and this appears to be up-regulated compared to noninvasive tumors (38), we probed whether the basal phosphorylation of PLCγ was due to EGFR signaling. Exposure of the cells to the EGFR kinase inhibitor PD153035 suppressed PLCγ phosphorylation (below). These data indicated that the EGFR-PLCγ signaling pathway was active in these cells, and provided evidence that this would be a target for limiting cell motility.

PLC Inhibition Reduces Migration Of 253J-B-V Cells. PLCγ signaling is required for increased migration signaled by a variety of growth factor receptors (6, 7, 39, 40). Since the ability to inhibit invasion through hindering cellular migration path ways is central to our hypothesis in these bladder cells, we subjected 253J-B-V cells to *in*

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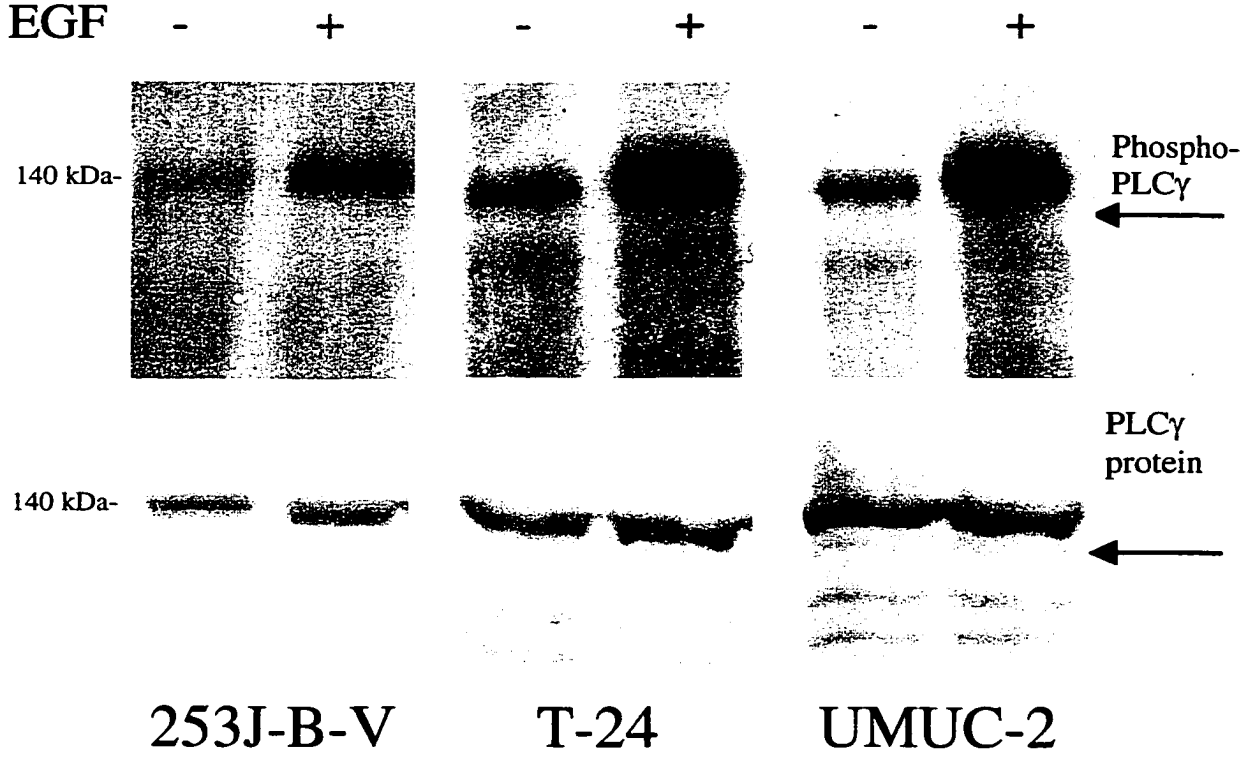

Fig. 1 PLCγ is active in bladder carcinoma cells and is responsive to EGF. *Top,* 253J-B-V, T-24, and UMUC-2 cells were grown to confluence, quiesced in media containing 1% dFCS for 24 h, and then treated with 10 nM EGF for 5 min. Cells were then washed, lysed, immonuprecipitated with antiphosphotyrosine (Transduction Labs; catalog #P11120) and Western blotted against anti-PLCγ (Upstate Biotechnology; catalog #05-163) as described in "Materials and Methods." *Bottom,* bladder cell lines were grown to confluence, quiesced in media containing 1% dFCS for 24 h, and then treated with 10 nM EGF for 5 min. Cells were then washed, lysed, immonuprecipitated with anti-PLCγ, and then Western blotted again with anti-PLCγ to show that protein levels remained constant in these cells despite EGF stimulation. Shown are representative blots of at least two experiments each.

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vitro wound healing assays. Cells were exposed to EGF with or without the PLC inhibitor U73122. As shown in Fig. 2, EGF treatment did not significantly enhance the rate of the wound closure over a 24-h period, due to postulated high autocrine signaling in these cells as evidenced by noticeable PLCγ phosphorylation in the absence of EGF (Fig. 1). As cells in this assay are confluent, thus highly conducive to autocrine signaling, it is possible that exogenously added EGF accelerates the rate of receptor internalization, thus causing an actual downshift in rate of wound closure. U73122 treatment, however, reduced migration to levels below that of nontreated cells.

PLC Inhibition Reduces Invasion of Bladder Carcinoma Cells Through Matrigel. In previous studies, the relevance of the PLCγ signaling pathway to tumor cell invasion was demonstrated by the ability of specific inhibitors to curtail invasion of prostate and breast carcinoma and glioblastoma cells (2, 14, 15). These findings were mirrored in the case of prostate cancer lines in *in vivo* studies in athymic nude mice. To determine whether this signaling pathway is rate-limiting for bladder cancer invasion, we plated 253J-B-V cells onto Matrigel-coated inserts in media containing 1% BSA. The target side of the insert contained complete media with 10% FCS, which was placed in the lower chamber. Cells were allowed to transmigrate in the presence or absence of 3 μM U73122 (as determined empirically at a level that does not exhibit nonspecific cellular toxicity on Matrigel) for a 72-h period. The 3-day period was chosen from prior published works (13, 16, 41) and the manufacturer's recommendations and was optimal for 253J-B-V cells treated with U73122. Invasion of cells that were treated with U73122 was reduced by ~38 ± 10% ($P < 0.05$) (Fig. 3*A*). T-24 and UMUC-2 cells were similarly

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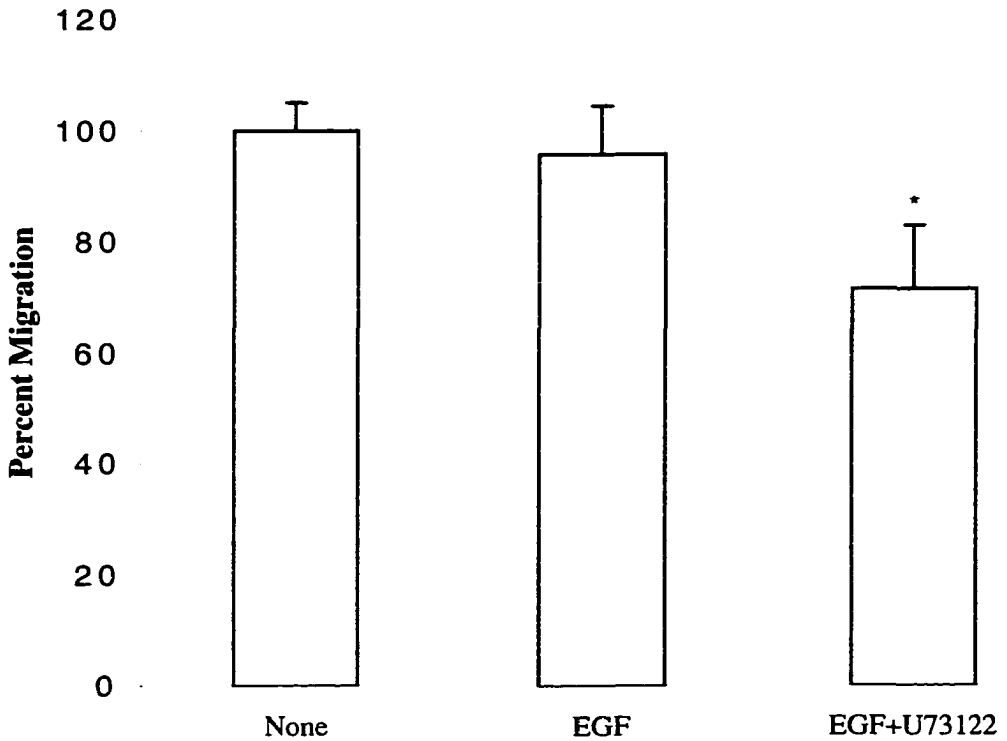

Fig. 2 U73122 retards cell migration during *in vitro* wound healing. 253J-B-V cells were grown to confluence and then placed in quiescence media containing 1% dialyzed FCS for 24 h prior to artificially wounding the monolayer with a rubber policeman. Cells were then treated with 10 nM EGF and/or 5 μM U73122 in appropriate wells and remained treated in quiescence media for another 24 h. The wound "gap" was measured by computer assisted microscopy at 0 and 24 h at three distinct points of the wound. All experiments were performed in duplicate wells in at least three separate experiments. *, $P < 0.05$ comparing EGF + U73122 treated *versus* untreated.

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subjected to Matrigel transmigration assays, with reductions in transmigration seen as a result of treatment. These results clearly validate the concept of PLCγ signaling as being a key component of invasion in bladder carcinoma cells.

As the observed effect could be due to U73122 affecting cell numbers and not limiting migration, we tested these cells for retarded proliferation by growing them on Matrigel-coated plates in the presence of U73122 and evaluating incorporation of thymidine as described under "Materials and Methods." As shown in Fig. 3*B*, cell counts were not significantly altered due to U73122 treatment. We utilized a PLCγ dominant negative construct, PLCz (5, 34), driven from a constitutively active SV40 early promoter (14, 16) to confirm that the inhibition seen with U73122 was due to PLCγ signaling disruption. Expressing the pXfPLCz construct in tumor cells mirrored the inhibitory effect of U73122 both *in vitro* and *in vivo* (13-16). We introduced the pXfPLCz construct into 253J-B-V cells, albeit transiently, with a ~ 50% electroporation efficiency as determined by co-electroporation with GFP (data not shown). As shown in Fig. 4, cells expressing pXfPLCz transmigrated the Matrigel cushion at a significantly reduced level. The convergent results of the U73122 and PLCz inhibitors demonstrated that the retarded invasion is due to specific inhibition of PLCγ in these cells. We separately tested the pXf promoter driving a control construct by transient expression; no discernible effect on invasion through Matrigel was noted (data not shown).

PLCγ Signaling is Secondary to EGFR Activity. As observed in our experiments, EGF treatment of 253J-B-V cells did not significantly alter their migration *in vitro* (Fig. 2). To determine whether this might be due to autocrine signaling through EGFR as suggested by basal PLCγ phosphorylation (Fig. 1), the effect of the EGFR-selective

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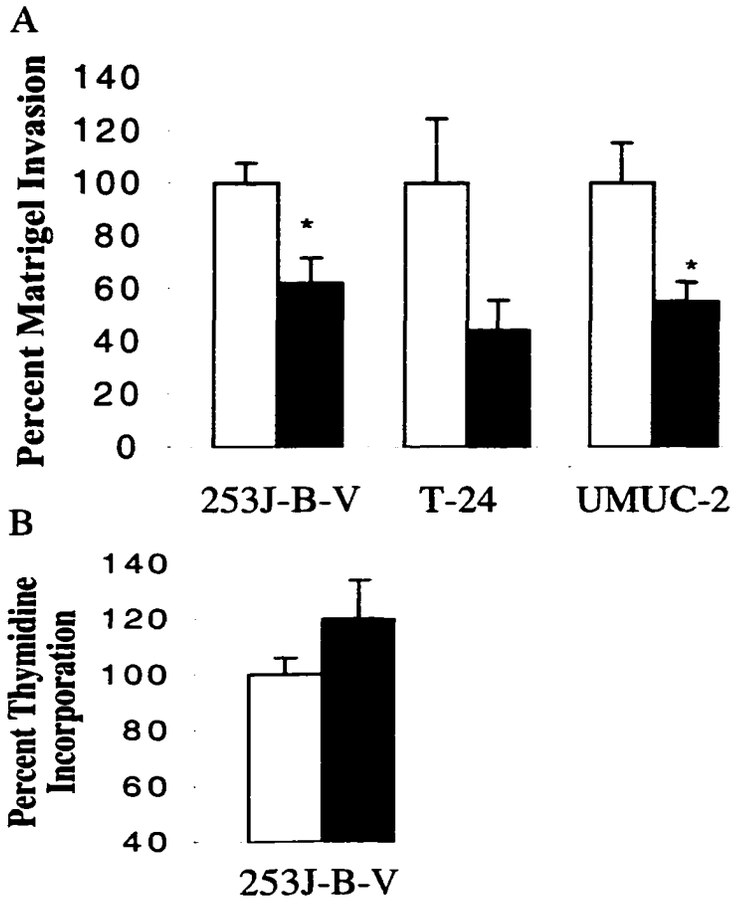

Fig. 3 U73122 retards cell invasion through Matrigel. *A*, 10,000 cells were plated in transwell plates containing a Matrigel-coated filter. The10,000 cells were plated on the topside in media containing 1% BSA and 3 μM U73122; the lower chamber contained complete media with 10% FCS as well as 3 μM U73122. The media in the upper chamber was replaced with serum-free media + 3 μM U73122 24 h later and maintained for a further 48 h (253J-B-V) or 24 h (T-24 and UMUC-2). Uninvaded cells were then removed from the upper chamber with a cotton swab, and the filter was stained with crystal violet and cells counted manually. *B*, cells were plated into 12-well plates, allowed to grow to confluence, and then placed in serum-free media for 48 h. Following this, appropriate wells were treated with 10 nM EGF and/or 3 μM of U73122 for a further 18 h. Five micro curies/ml [methyl-[^{3}H]] thymidine was added to each well, and incubation resumed for another 8 h. Wells were then washed with PBS followed by acid precipitation (5% TCA, 4°C for 30 min). The wells were washed with PBS and then treated with 0.2 N NaOH to solubilize the incorporated radiolabel. Scintillation counting quantitated the amount of incorporated thymidine. All experiments were performed in duplicate wells in at least three separate experiments. ■, U73122-treated cells; □, controls; *, $P < 0.05$ comparing U73122 treated cells *versus* untreated.

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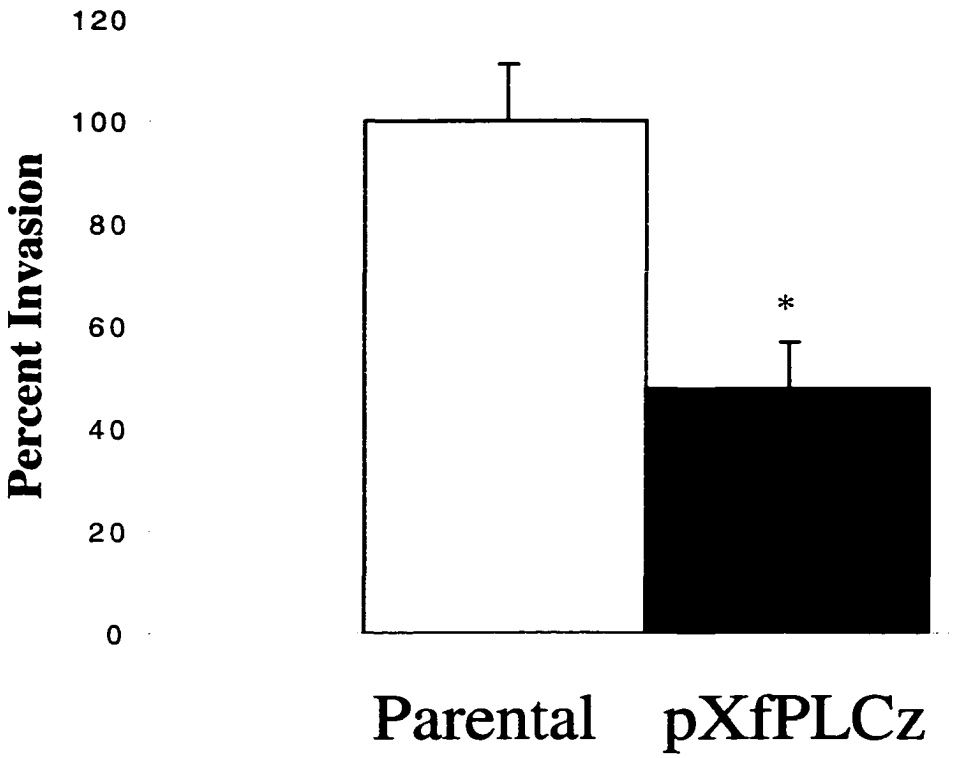

Fig. 4 Effect of PLCz on 253J-B-V cell invasion through Matrigel. PLCz was transiently expressed in 253J-B-V cells by pulse electroporation of the constitutively active pXfPLCz construct (separate co-GFP studies indicated a 50% expressing rate). Cells were plated into Matrigel invasion chambers as described in "Materials and Methods" (10,000 cells per well) and kept in media containing 1% BSA for the first 24 h followed by serum-free media for the remaining 48 h. The bottom chamber contained complete media with 10% FCS throughout. Uninvaded cells were then removed with a cotton swab, and cells on the underside were stained with crystal violet and counted. ■, PLCz-expressing cells; □, parental cells. All experiments were performed in duplicate wells in at least three separate experiments. *, $P < 0.05$ compared with parental cells

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inhibitor PD153035 was queried. PD153035 (500 nM) inhibited phosphorylation of EGFR and PLCγ significantly, as shown in Fig. 5*A*. PD153035 also reduced 253J-B-V migration by 75% (Fig. 5*B*). These findings support the contention that autocrine signaling contributes to bladder carcinoma cell movement. Furthermore, the finding that U73122 significantly reduced migration to levels below that of non-treatment exemplifies the notion that PLCγ is likely a convergence point of several key migration signaling enhancers whether autocrine or exogenous.

EGF Can Activate Calpain in 253J-B-V Cells. Despite the importance attached to the PLCγ motility pathway in the advent of tumor invasion and its emergence as a rate-limiting step, other molecular switches and pathways need to be equally considered (1, 2). Furthermore, the fact that EGFR inhibition decreased invasion to a greater extent than U73122 suggested that pathways and events in addition to PLCγ might be operative. Focal adhesion disassembly is a key modulator of cell migration, as inhibition of de-adhesion leads to cells remaining static despite protruding lammelipodia. Recently, the limited intracellular protease, calpain, has been shown to be integral in growth factor or integrin signaling of de-adhesion, independent of PLCγ (18, 20). To test the role of calpain in our cells, we first verified that calpain was active in 253J-B-V cells and inducible by EGF. We plated cells onto glass cover slips and treated without or with 2 μg/ml of the calpain inhibitor CI-1 (ALLN) and visualized calpain activity secondary to proteolysis of the synthetic pro-fluorescent substrate, Boc-LM-CMAC. As shown in Fig. 6, EGF treatment notably increased calpain activity as demonstrated by increased fluorescence, while CI-1 treatment abrogated this activity.

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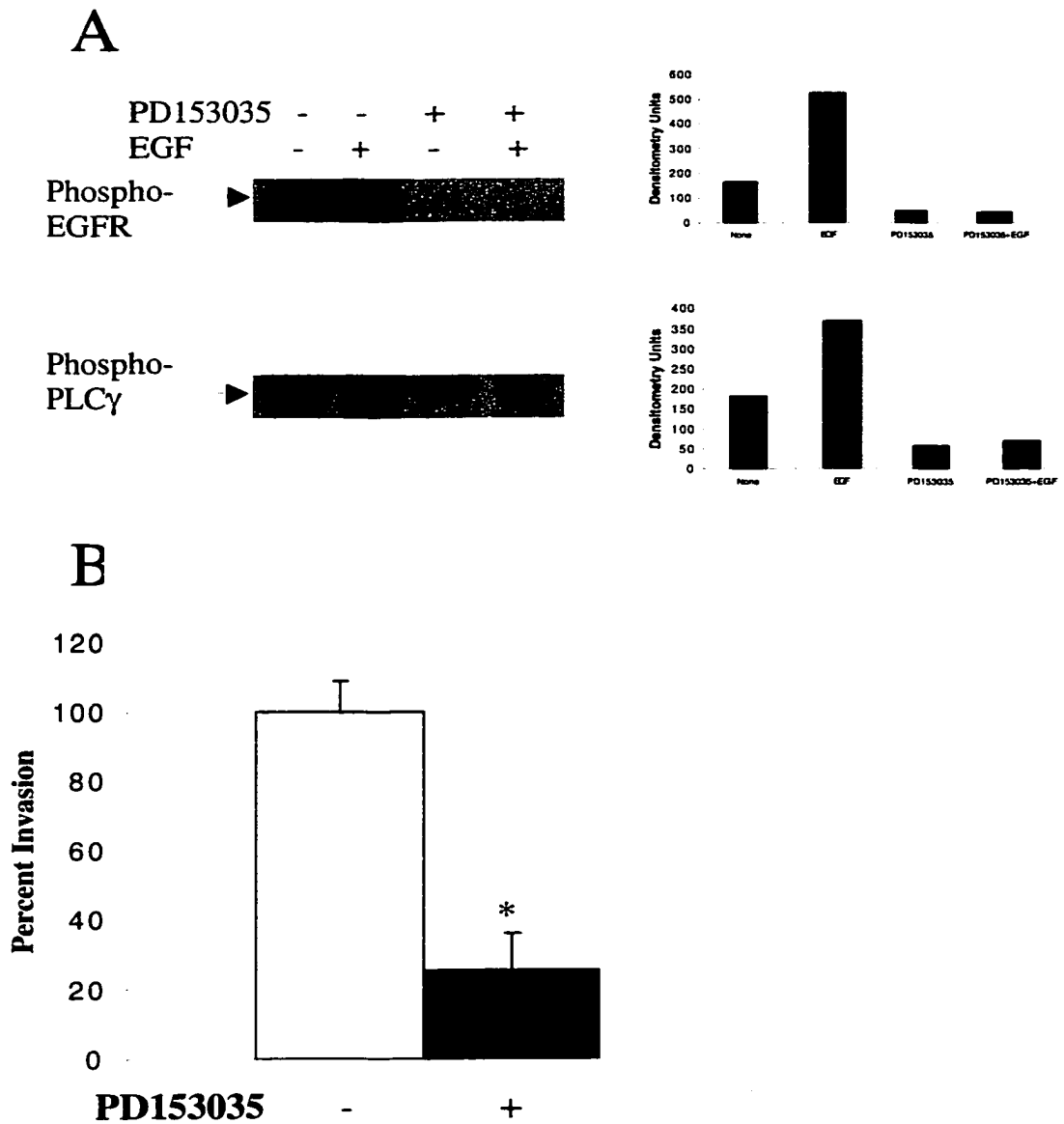

Fig. 5 Effect of PD153035, an EGFR kinase inhibitor, on cell invasion through Matrigel. *A*, EGFR and PLCγ phosphorylation in 253J-B-V cells is inhibited by PD153035. Cells were starved in media containing 1% dialyzed FCS for 24 h after which plates were treated with 500 nM of PD153035 for 25 min at 37°C followed by 10 nM EGF for a further 5 min. Lysates were separated by SDS-PAGE and Western blotted with anti-phosphotyrosine antibody. Densitometric analysis was performed on computer scanned images of the blots using the NIH Image application. *B*, PD153035 limits 253J-B-V cell invasion. Cells were evaluated for transmigration through a layer of Matrigel as described previously, using 500 nM of PD153035 for a 48-h period. The10,000 cells were plated per well. ■, treated cells; □, untreated cells. All experiments were performed in duplicate wells in at least three separate experiments. *, $P < 0.05$ comparing PD153035 treated cells *versus* untreated.

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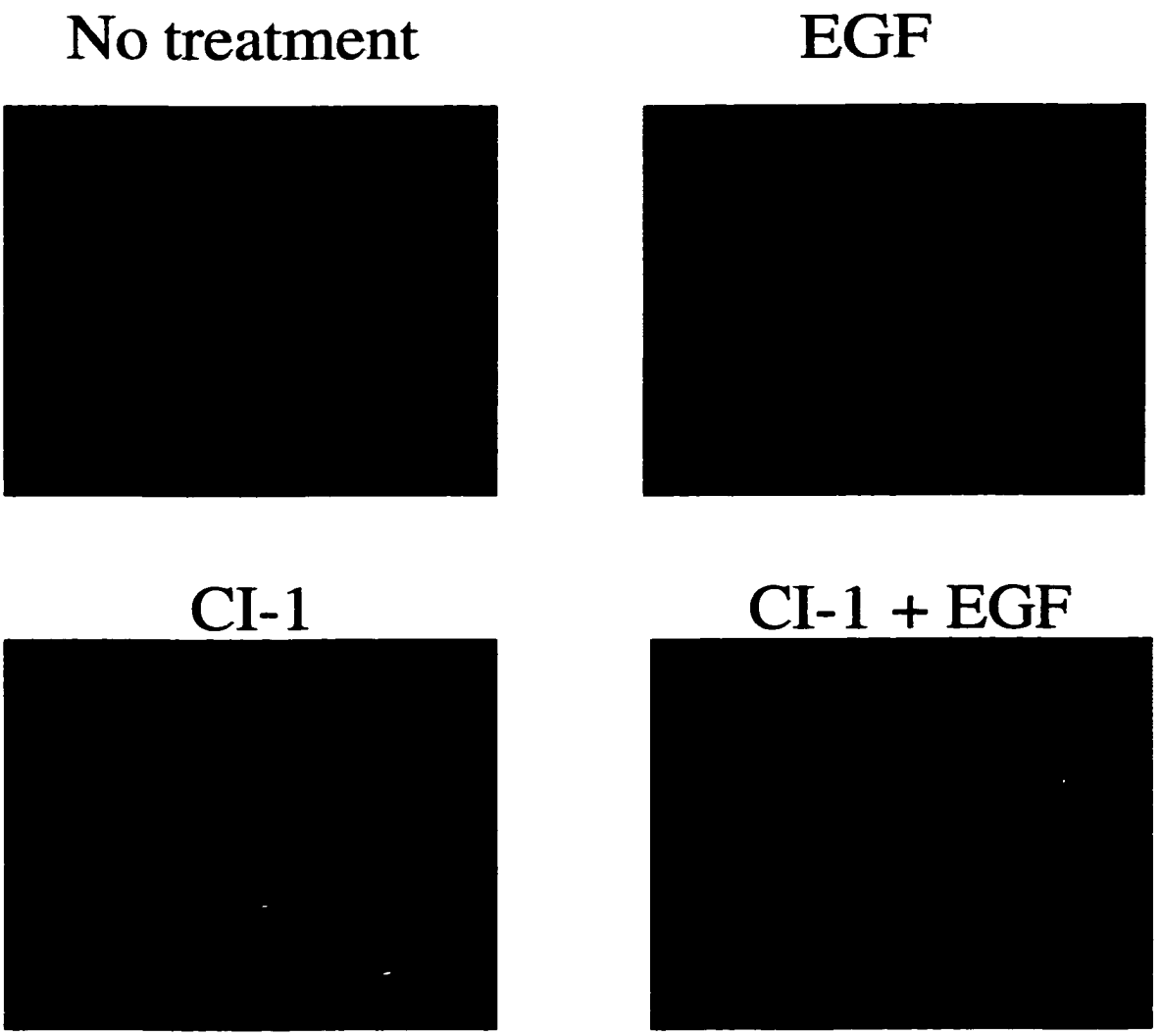

Fig. 6 Calpain activity in 253J-B-V cells and inhibition by CI-1 (ALLN). Cells were plated on wells containing glass cover slips and incubated in quiescence media containing 1% dialyzed FCS for 24 h. Wells were then treated with 2 μg/ml CI-1 (ALLN) for 1 h. Forty minutes into the treatment, 0.5 μM of Boc-LM-CMAC was added to all wells, followed by 10 nM of EGF 15 min later for a 5-min EGF stimulation. Cover slips were then removed from the media and mounted onto glass slides and photographed under a fluorescent microscope. Shown are representative photographs of two independent experiments.

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Inhibition of Calpain Limits Migration and Invasion of Bladder Carcinoma Cells. As described previously, a key process in cell migration is focal adhesion disassembly and cell detachment, which is a process in which the molecule calpain plays a key role. To test the applicability of this in 253J-B-V cells, we performed *in vitro* wound healing assays in the presence of 2 μg/ml CI-1. As shown in Fig. 7*A*, CI-1 treatment significantly inhibited cell migration. This suggested that if, as proposed, motility was critical for tumor cell invasion, inhibition of calpain should reduce invasion similarly to PLCγ inhibition. We performed Matrigel transmigration assays in the presence or absence of the calpain inhibitor CI-I. 253J-B-V cells treated with 2 μg/ml CI-1 showed markedly reduced invasion through Matrigel (Fig. 7*B*). These studies strongly suggest that, while PLCγ is a key regulator of invasion, calpain is also a separate rate-limiting molecular switch, leading to the conclusion that it is the whole process of migration per se that collectively produces the invasive phenotype.

DISCUSSION

Tumor invasion is a multistep process wherein cells have to first recognize the extracellular matrix (ECM), reorganize the ECM, and migrate through it to adjacent environments. Abrogation of any of these events would be rate-limiting for tumor progression. Recently, many investigators, including us, have targeted select molecular controls of these events to limit tumor invasion (reviewed in Refs 2, 42, 43). These individual studies have demonstrated that inhibition of specific signaling cascades can block tumor cell invasion *in vitro* and *in vivo*. However, the causal relationship is often indirect due to both the pleiotropic nature of signaling cascades and the challenges inherent in visualizing invasion (44, 45). Thus, we have sought to determine whether

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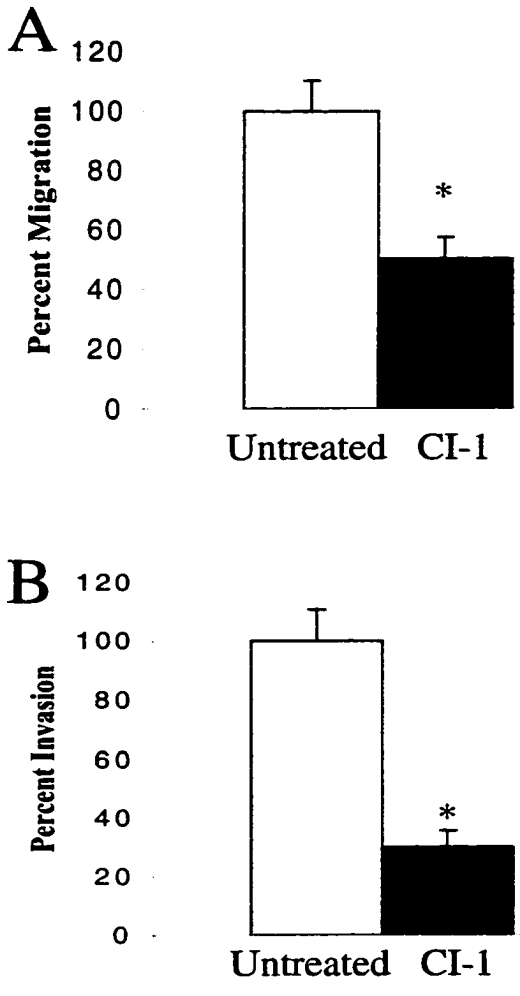

Fig. 7 Effect of inhibiting calpain on bladder cell migration and invasion. *A*, cells were grown to confluence in 6-well plates and then placed in media containing 1% dialyzed FCS for 24 h. The monolayer was then "wounded" with a rubber policeman. After wounding, cells were placed in 1% dFCS media containing mitomycin-C (0.5 μg/ml) and some wells treated with 2 μg/ml CI-1. Using a microscope, the wound area was photographed initially and after 24 h. The distance that cells had traveled into the denuded area was determined using computer-driven image analyses, at three distinct points in the wound, and averaged. ■, treated cells; □, untreated cells; *, $P < 0.05$. *B*, 253J-B-V cells were evaluated for transmigration through a layer of Matrigel as described previously. Ten thousand cells were plated in the upper chamber of the transwell plate in media containing 1% BSA and 2 μg/ml calpain inhibitor-1 (CI-1). After 24 h the media in the top chamber was replaced with serum-free media also containing 2 μg/ml CI-1 for a further 24 h. The bottom chamber contained complete media with 10% FCS and 2 μg/ml CI-1 throughout. ■, treated cells; □, untreated cells. All experiments were performed in duplicate wells in at least three separate experiments. *, $P < 0.05$ comparing CI-1 treated cells *versus* untreated.

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dysregulated cell motility per se is required for tumor invasion by investigating the effects of blocking two distinct arms of the motility pathway in a hitherto unexamined tumor type, bladder carcinoma. This was approached by attacking two key switches, PLCγ and calpain, that control distinct aspects of cell motility, cytoskeletal reorganization and rear detachment, respectively.

While invasion can be limited by blocking any of the required events (matrix recognition, matrix degradation, and motility), a central question is which event is actually dysregulated to promote invasion. We, and others, have proposed that it is inappropriate induction of cell motility that leads to tumor invasion. Most cancers exhibit autocrine signaling through growth factor receptors that are often up-regulated, and the most commonly up-regulated receptor is EGFR. Bladder carcinomas present potential autocrine signaling through EGFR and other receptors which correlates with tumor invasion through the bladder muscle layer (46-48). These tumors provide a system with which to test the effects of abrogating tumor cell motility. As human prostate carcinoma lines, we used the highly invasive and metastatic variant of 253J cells, 253J-B-V (31), with confirmation from T-24 and UMUC-2 cells. Both of the targeted pathways were functional in this cell line. Abrogation by pharmacological or molecular inhibitors of either pathway similarly blocked tumor invasion. These findings point to motility being the critical element that can be blocked.

Initially, as PLCγ is a key component of cellular migration (5, 6, 49), we asked what effect PLC inhibition would have on *in vitro* cellular migration The PLC inhibitor U73122 reduced migration to a level below that of nontreated cells. These data support the contention that autocrine signaling from EGFR and other receptors collectively promote motility via PLCγ, thus promoting PLCγ as a key point of signaling convergence in

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increased tumor progression (1). Therefore, PLCγ is a target molecule for inhibition of cellular migration. Based on previous studies, PLCγ signaling is hypothesized to be rate-limiting for tumor cell invasion. This was borne out by inhibition of transmigration of Matrigel by U73122 or the dominant-negative construct PLCz. This inhibition supported earlier reports from prostate and breast cancers and glioblastomas (14-16). Despite these findings, the conclusion that motility is the only target of these interventions has been brought into question by finding specific gene expression alterations downstream of PLCγ signaling (17). Thus, while PLCγ remains a proven critical element in tumor invasion, the full contribution of epigenetic motility signals remains uncertain.

A second signaling pathway that modulates a distinct aspect of cell motility has recently emerged. The limited intracellular protease, calpain, has been shown to be required for movement across moderately to highly adhesive substrata such as ECM (18-21). While the mechanism by which calpain is activated during haptotactic motility is speculative (23, 50), growth factor receptors trigger the M-calpain isoform downstream of the ERK/MAPK pathway (18). Blocking of calpain by the selective pharmacological agent CI-I limited tumor cell invasion to a similar extent as PLCγ inhibition. We did not assess the effects of both inhibitors together since (a) inhibition of Matrigel transmigration by CI-I was already >75%, and (b) because the PLC inhibitors were used at concentrations that only partially inhibit PLC signaling [to avoid extraneous toxicity (5)], it would be difficult to distinguish additive effects suggestive of a secondary event. Thus, as these two distinct inhibitory strategies produced similarly diminished invasion, we concluded that cell motility per se is a feasible target to limit tumor invasion.

The designation of the biophysical process of motility as a possible therapeutic target carries a number of implications. The usual approach is to define specific mo-

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lecular targets, which are attractive in testing and designing lead compounds. However, the individual targets might either prove refractory to inhibition or be distributed in normal tissue such as to not provide for a therapeutic index. By identifying a process, a larger number of potential targets become available. For motility these would include modulators of new distal adhesions, such as focal adhesion kinase (FAK) and integrin-linked kinase (ILK) (51, 52), and cytoskeletal protrusion, such as the Rho family of small GTPase (53). This would allow for one to screen numerous inhibitors for a more therapeutically amenable target or even use a cocktail of subtherapeutic compounds to minimize toxicities.

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INVASIVENESS OF DU-145 HUMAN PROSTATE CARCINOMA CELLS EXPRESSING DIFFERENTIALLY ACTIVATED PLCγ INVOLVES ALTERED uPAR EXPRESSION

by

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In preparation for *Cancer Research*

Format adapted for dissertation

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ABSTRACT

Cell migration is a critical rate-limiting step in tumor invasion. A number of previous studies have implicated a series of signaling pathways in tumor cell migration, and blocking these pathways significantly reduces invasion both *in vitro* and *in vivo*. Phospholipase C (PLC)γ is a pivotal molecule for cellular migration induced by epidermal growth factor receptor (EGFR) stimulation. Our previous studies have established that PLCγ is critical for tumor cell invasion both through *in vitro* extracellular matrices and *in vivo* in mice. However, still unknown are the genomic changes that accompany PLCγ activity, which occur during extended signaling by PLCγ beyond the initial phase. To investigate this, we utilized three sublines of the DU-145 human prostate carcinoma cell line that were engineered to differently activate PLCγ and were variously invasive. We subjected these sublines to mRNA microarray analysis of 9,216 random gene expression tags. We found that the urokinase-type plasminogen activator receptor (uPAR) is the highest overexpressed transcript in the highly invasive EGFR-overexpressing DU-145 WT cells and is down-regulated in the noninvasive PLCγ signaling-restricted DU-145 c'973 cells. To expand on the notion of uPAR being an invasion promoter, we constructed uPAR cDNA in both sense and antisense positions in a constitutively active promoter and stably expressed the constructs in appropriate DU-145 sublines. Antisense to uPAR significantly restricted invasion of the highly invasive DU-145 WT cells through Matrigel and reduced aggressiveness of tumors in nude mice. Also, up-regulating uPAR significantly increased the invasiveness of DU-145 parental cells in Matrigel. These results provide a causal link between EGFR-modulated migration via PLCγ and

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invasion on one hand, and the various cellular functions that come into play as a result of this on the other.

INTRODUCTION

One of the rate-limiting steps of tumor cell invasion is its ability to migrate through a dissolved or rearranged extracellular matrix (ECM). An increasing amount of recent data not only have elucidated a variety of cellular pathways and mechanisms involved but also have alluded to the interdependence and cross-communication of many of these pathways. However, one relatively common aspect of cellular invasion mechanisms is their mediation by surface receptors which, in transformed cell, are often over-expressed and up-regulated. Of these receptors, EGFR is one of the most frequently up-regulated in tumors (1). In our previous studies utilizing DU-145 prostate carcinoma cells with up-regulated EGFR, we identified the PLCγ-mediated cell migration pathway as being rate-limiting for tumor cell invasion (2-5). PLCγ is a convergence point for many growth factor receptors which, through its SH2-mediated association with activated growth factor receptors, is phosphorylated and activated (6-9). Since many such growth factor receptors, such as EGFR (10-12), are up-regulated in tumor cells and present autocrine loops, it follows that PLCγ activity is also enhanced in such systems.

PLCγ activation is required for growth factor-induced migration via such molecules as EGFR (13), platelet-derived growth factor receptor (PDGFR) (14), and insulin-like growth factor-I receptor (IGF-IR) (15). We previously showed that human prostate carcinoma DU-145 cells overexpressing full length EGFR (WT DU-145) promoted invasion of these cells both *in vitro* and *in vivo* (4, 16), while expression of a truncated

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EGFR, which is fully mitogenic but unable to activate PLCγ, drastically reduced these cells' invasiveness (4, 16). To ascertain whether this effect was due to EGFR signaling of PLCγ, we performed further studies in which inhibition of PLCγ, either pharmacologically or molecularly, resulted in a significant decrease of tumor invasion in these cells (3, 16) and other prostate and breast carcinoma lines (2). This requirement for PLCγ in tumor invasion has been confirmed in other tumor types (17, 18). These findings established a solid role for growth factor receptor induction of the PLCγ migration pathway in tumor cell invasion.

The general model for PLCγ-mediated motility is an epigenetic one of membrane-localized phosphotidlyinositol bisphosphate (PIP_2) hydrolysis and mobilization of actin binding proteins (19, 20). However, in more prolonged motility during invasion, secondary proteomic changes may come into play (21-23). To evaluate the gene expression changes that occur in our variably invasive DU-145 sublines, we tested mRNA samples from each by microarray analysis and compared the expression of 9,216 genes among the samples. We discovered a handful of genes that were up-regulated in the more invasive WT DU-145 cells and another handful that were up-regulated in the relatively uninvasive c'973 DU-145 cells (24). Of interest to our study was uPAR, which was the most up-regulated gene in the invasive WT DU-145 cells.

uPAR has been implicated as a major player in promoting tumor invasion in a variety of cancers such as glioblastoma (25) and bladder carcinoma (26). There are a number of possible mechanism by which this occurs. The heavily glycosylated uPAR, when binding its ligand, urokinase-type plasminogen activator (uPA), serves to convert plasminogen to plasmin, which in turn actively dissolves ECM components. uPA may also

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convert pro-hepatocyte growth factor (HGF) to its active form (27), which is intriguing because HGF levels are also up-regulated in the highly invasive WT DU-145 cells. uPAR also is involved in cytoskeletal rearrangement and activation of various other downstream molecules (reviewed in Ref. 28). Since both ECM manipulation by tumor cells and cellular migration are a hallmark of invasion, a highly active role for uPAR in tumor cell invasion becomes immediately recognizable.

Because this protein topped the list of up-regulated genes in the EGFR/PLCγ overactive and highly invasive WT DU-145 cells, we asked whether uPAR itself was secondary to increased PLCγ activity or was in itself an associated but independent rate-limiting factor in tumor cell invasion. We constructed a full-length uPAR cDNA fragment and inserted it in both sense and antisense positions into a vector carrying a constitutively active promoter and introduced these constructs into our DU-145 sublines. We demonstrated that elevating uPAR expression in parental DU-145 cells increased their invasiveness through Matrigel to levels similar to the EGFR overexpressing WT DU-145 cells, while the highly invasive WT DU-145 cells' invasiveness was retarded by the antisense uPAR cDNA fragment. In order to establish whether this behavior is reproducible *in vivo*, we injected the highly invasive WT DU-145 cells expressing antisense uPAR cDNA into athymic nude mice; and while control WT DU-145 cells formed highly invasive tumors, the antisense-carrying cells were significantly less aggressive and were reduced in diaphragm invasiveness. These results indicate that uPAR plays a rate-limiting role in growth factor-induced tumor cell invasion.

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MATERIALS AND METHODS

Animals. Male athymic BALB/c *nu/nu* mice, 6-8 weeks of age, were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development (Frederick, MD) and housed in suitable conditions. Mouse weights ranged from 20-30 g at onset of experiments.

Cell Culture. WT DU-145 and c'973 DU-145 prostate carcinoma cells were generated as described previously (4). WT cells overexpress EGFR to levels that over-saturate the capacity of the degradation pathway and therefore do not undergo autocrine-induced down-regulation. EGFR in c'973 cells are truncated at that amino acid and are missing their internalization and PLCγ-signaling domains but are fully mitogenic (13, 29). The cells were maintained in DMEM (4.5 g/ml glucose) containing 10% FCS and supplemented with L-glutamine (2 mM), penicillin/streptomycin (100 units/ml), nones-sential amino acids (0.1 mM), and sodium pyruvate (1 mM). For stable selection of WT or c'973 EGFR, cells were grown in G418 (1,000 μg/ml). Sense and antisense uPAR constructs were introduced into the cells by electroporation. Briefly, uPAR was cloned into the constitutively active pXf vector, which contains the SV40 early promoter (13), in either sense or antisense directions. The sense uPAR gene was cloned via PCR with the *Xba* I restriction site at the 5' end and the *Hind* III site at the 3' end, while the antisense gene was cloned by switching the positions of these two restriction enzymes. Stable transfectant cells were selected by additionally supplementing the above media with 1.2 μg/ml methotrexate.

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Tumor Cell Inoculations. We utilized the intraperitoneal xenograft model for invasiveness as determined by diaphragmatic invasion (3, 16). Cells were suspended in culture media and injected (26.5-gauge needle) into the peritoneal cavity (2 million cells in 200 μl total volume). Mice were asphyxiated after 60 days with CO_2 and necropsied, with removal of the pancreas, spleen, kidneys, diaphragm, lungs, and any tumorous tissue. All tissues were fixed in 10% buffered formalin, paraffin embedded, serially sectioned, and stained with hematoxylin and eosin (H&E). Invasion of the diaphragm was determined by microscopic quantitation. The protocol was approved by the Pittsburgh Veteran Administration Medical Center Institutional Animal Care and Use Committee.

Immunoblotting. Cells were grown in 6-well plates to semiconfluence, washed with PBS, and lysed with Laemelli buffer. The lysate was separated by SDS-PAGE and immunoblotted. Primary antibodies used included anti-uPAR (American Diagnostica; catalog #3936) and anti-actin (Sigma; catalog #A 2066) The staining was visualized by a secondary antibody linked to alkaline phosphatase or horseradish peroxidase.

Transmigration Assays. Cell invasiveness *in vitro* was determined by the ability to transmigrate a layer of ECM, Matrigel, in a modified Boyden chamber assay (30, 31). Matrigel invasion chamber plates were obtained from Becton Dickinson/Biocoat (catalog # 40480). For each experiment, 10,000 cells were plated randomly and distributed among plates with different lot numbers, with each experiment performed in duplicate or triplicate. Despite possible variances in EGFR ligand concentrations in Matrigel, these concentrations are saturating since even "reduced growth factor Matrigel" contain relatively

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high amounts of EGF (up to 0.5 ng/ml compared to 0.5-1.3 ng/ml in regular matrigel) (Becton Dickinson Labware 1997/98 catalog description, page 128). Cells were kept in serum-free media containing 1% BSA for the first 24 h and then replaced with serum-free media for the remaining 24 h. Enumeration of the cells that invaded through the matrix over a 48-h period was accomplished by removing uninvaded cells with a cotton swab and then fixing, staining with crystal violet, and visually counting cells on the bottom of the membrane.

Microarray Construction. Microarray slides were prepared as described (32-34). Total RNA was extracted using the Trizol reagent (Life Technologies, Inc.) according to manufacturer's instruction. Poly(A)+ RNA was purified using the Oligotex-dT mRNA batch kit from Qiagen. Labeling and hybridization were performed as described (32-34). Briefly, first strand DNA probes were generated by incorporation of Cy3-dCTP or Cy5-dCTP (American Pharmacia) during reverse transcription of 1 μg purified mRNA. The resulting cDNA probes were purified by vacuum filtration, denatured at 94°C, and hybridized to an arrayed slide overnight at 42°C. Slides were washed in 1 x SSC/0.2% SDS for 10 min, and then in 0.1% x SSC/0.2% SDS for 20 min. Slides were rinsed and dried, and fluorescence was captured using the *Avalanche* dual laser confocal scanner (Molecular Dynamics). Fluorescent intensities were quantified using Arrayvision 4.0 (Imaging Research).

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RESULTS

uPAR Transcript Levels Correlate With Increased PLCγ Activity. One of the critical mechanisms of tumor cell invasion is its migratory ability as determined by growth factor receptor-induced activation of the PLCγ pathway (13). In our previous studies, DU-145 cells with overexpressed EGFR (WT DU-145) invaded through a human ECM significantly further than parental cells, whereas the fully mitogenic but PLCγ signaling-impaired c'973 DU-145 cells were relatively noninvasive in such *in vitro* assays (4). These observations were mirrored *in vivo*, where WT DU-145 cells obliterated the diaphragms of athymic nude mice, while c'973 DU-145 cells were essentially noninvasive (16). These results clearly defined a role for PLCγ as a key modulator of tumor cell invasion, especially as these observations were verified in a number of other cell lines, including breast (2), colon (35), and glioblastoma (18). However, one remaining underlying question is what gene expression changes accompany up-regulation of PLCγ activity.

Numerous changes and modification of gene expression coincide with altered oncoprotein expression during oncogenic transformation (23, 36). To this end, we subjected mRNA from all three DU-145 sublines to microarray analysis where the transcriptional activity of 9,216 genes were compared among each subline. Of these, a handful were up-regulated in the order WT DU-145 > parental DU-145 > c'973 DU-145, while a separate group was up-regulated in the reverse order being highest in c'973 DU-145 (Table 1). The gene products up-regulated in the highly invasive WT DU-145 cells were deemed invasion promoters, while those up-regulated in the relatively noninvasive c'973 DU-145 cells were deemed invasion supressors. uPAR topped the list of invasion pro-

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Table 1 List of Genes Identified By Microarray Analysis That Parallel the Invasive Phenotype of DU-145 Cells

PLCγ-regulated genes	Candidate promoter or suppressor Promoter = WT > Parental > c'973 Suppressor = WT < Parental < c'973
Urokinase Receptor	Promoter
Insulin-like Growth Factor I	Promoter
Hepatocyte Growth Factor	Promoter
PDGF-A	Promoter
Cyclin I	Promoter
Integrin αv	Promoter
EST AA207900	Promoter
EST 548375	Promoter
70kD Heat Shock Protein	Suppressor
MHC Class I	Suppressor
EST AA548375	Suppressor
EST AA047755 (Prostin-1/DPM3)	Suppressor

moters (Fig. 1). This protein has already been implicated in enhancing tumor progression (37, 38) and signaling motility (39, 40), and its apparent link to PLCγ-modulated invasion is intriguing.

uPAR Protein Levels Are Upregulated in Invasive WT DU-145 Cells . The microarray data related the variation of uPAR mRNA transcripts among the DU-145 sublines; it is prudent to assume that a multitude of post-transcriptional events may not yield a proportional protein content. Alternative splicing, post-transcriptional modifica

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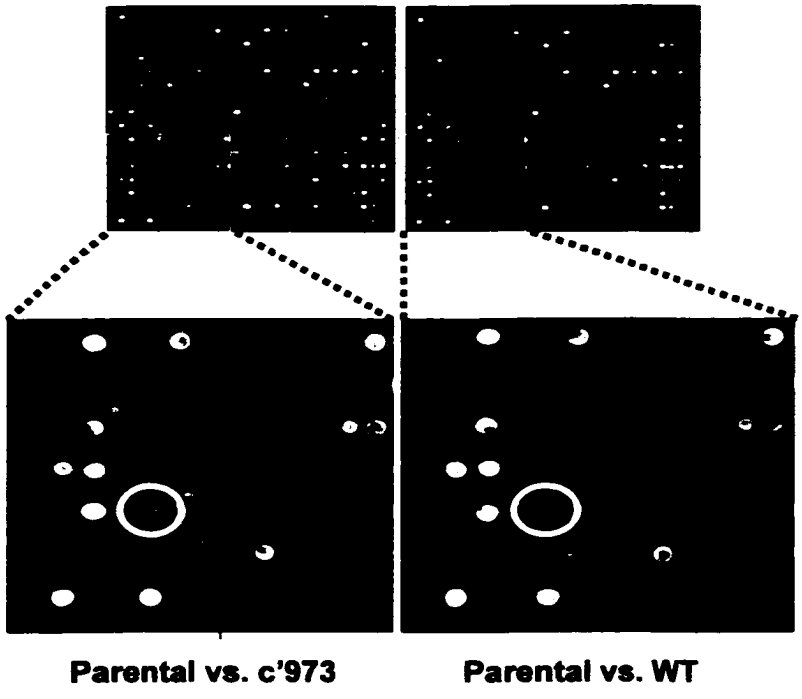

Fig. 1 Microarray analysis of mRNA from three DU-145 sublines. mRNA was extracted from the three DU-145 sublines and tested for differential regulation of 9,216 candidate genes. The left magnified panel indicates up-regulated uPAR mRNA in PA DU-145 cells compared to c'973 DU-145 (green), whereas the right panel indicates down-regulated uPAR in PA DU-145 cells compared to WT DU-145.

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tion, lack of GPI anchor addition, or proteolitic cleavage of the GPI anchor may influence the expression of uPAR on the cell surface (41). We obtained cell lysates of the three DU-145 sublines and subjected them to immunoprecipitation of uPAR followed by immunoblotting for uPAR. The purpose of immunoprecipitating first was primarily for clarity. As proteolitic cleavage of uPAR is a very common event on cell surfaces, a combination of antiproteases were used to treat the cells while lysing: 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) and phenylmethanesulfonyl fluoride (PMSF). As shown in Fig. 2, uPAR protein levels were indeed higher in WT DU-145 than parental. Notably, although not as high as WT DU-145, uPAR protein levels were also higher in c'973 DU-145 than in parental lines. This phenomenon may not actually be very surprising considering the cellular processes that occur regarding uPAR and the strong possibility that lack of PLCγ activity in these cells results in uncleaved GPI anchors and a greater concentration of uPAR remaining on the cell surface as a result. A point supporting this is that the most prominent invasion suppressor is the regulatory subunit of a rate-limiting step in GPI anchors, dolichol-phosphate-mannose-3 (DPM3) (24, 42). These results indicate a discrepancy of uPAR levels among the three sublines that correlate with the mRNA levels, as seen if Fig. 1, therefore giving credence to an active role for uPAR in tumor cell progression in concert with other cellular events that promote cell migration.

Modulation of uPAR Expression Correlates With Invasiveness Through Matrigel. The above studies suggested that uPAR expression may either enhance or be required for invasion. To determine the requirement for uPAR in tumor cell invasion, we selected DU-145 cells overexpressing the full length EGFR WT (WT DU-145) since

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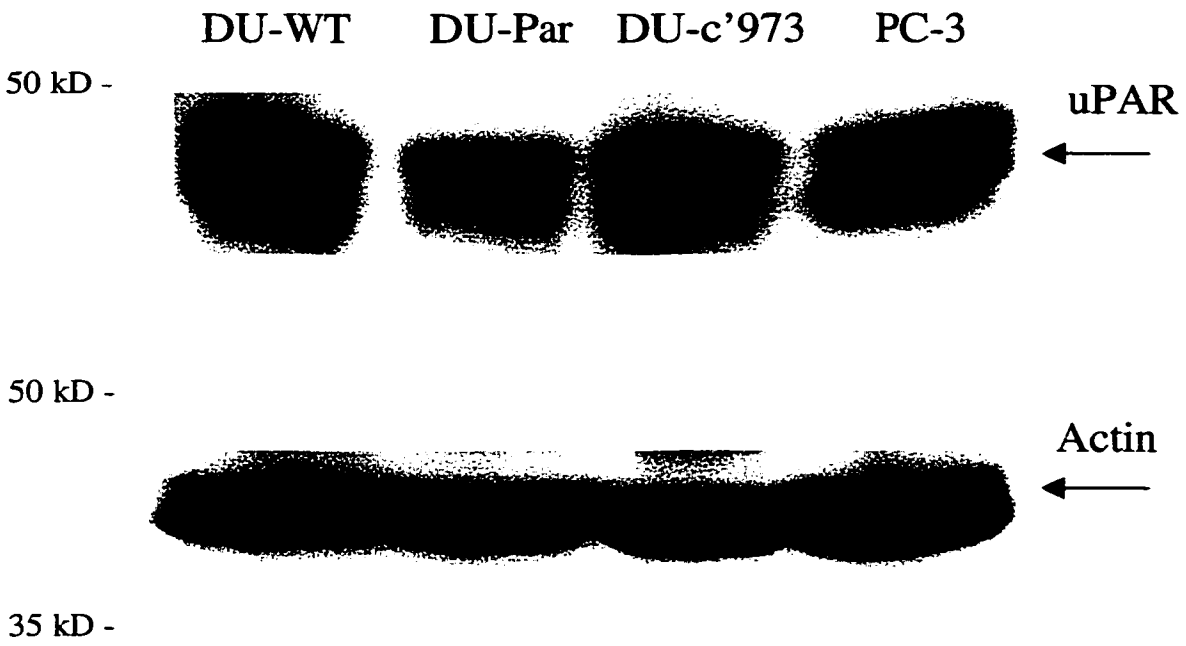

Fig. 2 Expression of uPAR protein in DU-145 sublines. Whole cell lysates from each of DU-145 wild-type, parental, and c'973, as well as for a separate prostate cancer cell line (PC-3), were collected and quantitated using the BioRad protein assay. Identical concentrations of lysates were then separated in 10% SDS PAGE and blotted with anti-uPAR antibody (American Diagnostica; catalog #3936) under non-reducing conditions. Concurrent blotting of an identical gel with anti-actin (Sigma; catalog #A-2066) was also done to show equal protein loading. Shown is a representative of four blots

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these cells are significantly more invasive than parental cells both *in vitro* and *in vivo* (4, 16). To determine if uPAR expression enhances invasiveness, we utilized the moderately invasive parental line. As PLCγ is required for motility, we did not challenge the noninvasive c'973 DU-145 cells in these assays, because the lack of one required element would dominantly mask the possible contribution of any cofactors such as uPAR. WT DU-145 and c'973 DU-145 cells are syngenic with parental cells with the exception of an extra EGFR cDNA (full length or truncated) driven from a retrovirus long-terminal repeat (LTR) cassette (29). WT DU-145 cells also presented overexpressed uPAR mRNA and protein. To study the effects of uPAR down-regulation in aggressively invasive cells, we cloned a full length uPAR cDNA fragment (including the GPI anchoring domain) downstream from a constitutively active SV40 early promoter in the eukaryotic expression vector pXf. Stable selection of the electroporated construct was accomplished using 1.2 μg/ml methotrexate in the media. As shown in Fig. 3, Western blot analysis of cell lysates indicated an increased expression of uPAR protein in the construct-expressing cells. Similarly, we cloned the uPAR cDNA fragment in the antisense direction into the pXf promoter and stably selected the construct in the less invasive parental DU-145 cells and noninvasive c'973 DU-145 cells. As expected, uPAR protein levels were reduced in cells expressing the antisense cDNA (Fig. 3). These findings are significant to our study since uPAR is generally actively cleaved at the GPI anchor and secreted (43), thereby reducing levels at the cell surface. It remains unknown whether up- or down-regulation of uPAR causes a concomitant increase or decrease in rate of cleavage to compensate. As these data indicate, both sense and antisense cDNA constructs established the desired effects of stably increasing or decreasing uPAR levels. Our data already confirmed the

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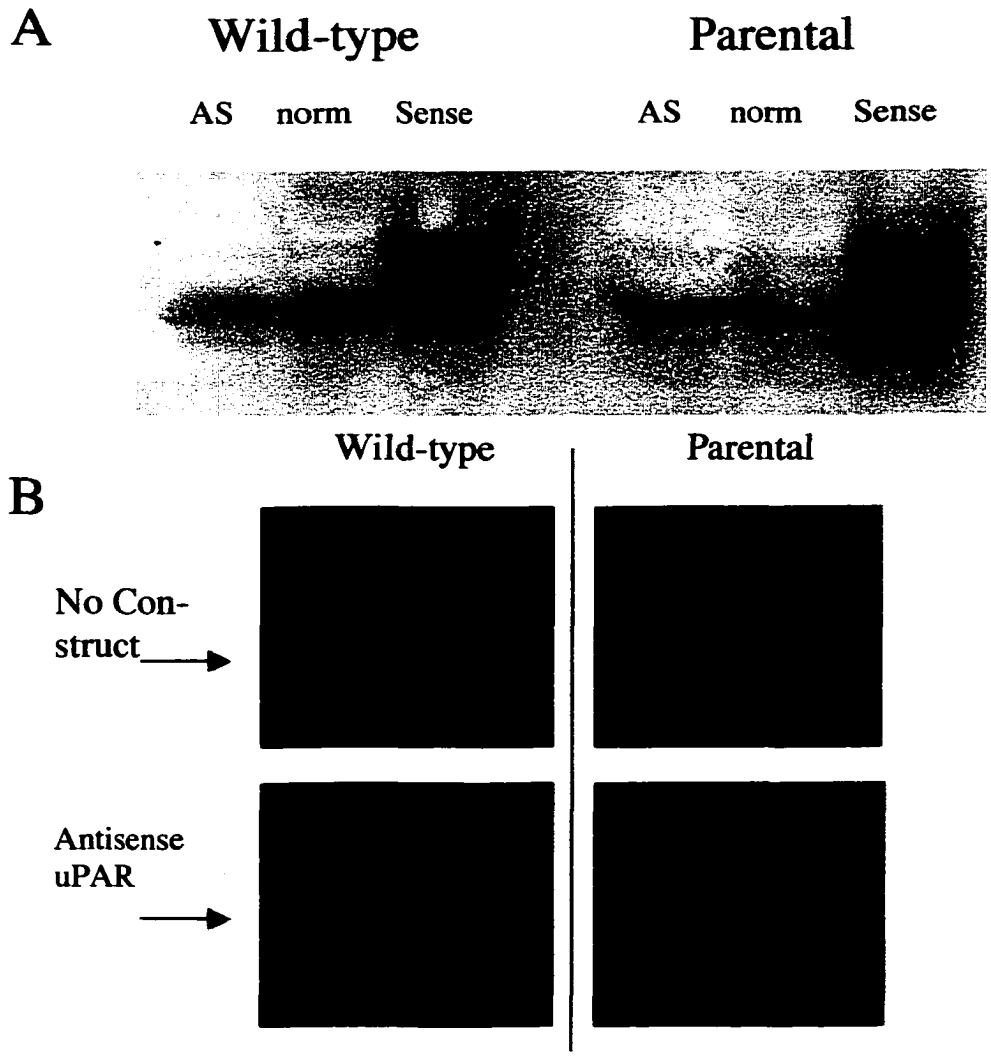

Fig. 3 Expression of sense or antisense uPAR cDNA fragments in DU-145 sublines. Primers were designed to add 5' *Hind* III and 3' *Xba* I restriction sites to uPAR active region cDNA via PCR. Proper and reverse antisense uPAR cDNA was produced via PCR. The fragments were then ligated into the pXf vector, which contains a constitutively active SV-40 promoter. The constructs were electroporated into each of the strains of DU-145 cells and selected with methotrexate. uPAR cDNA was provided courtesy of Abbott Laboratories. *A*, DU-145 cells expressing sense or antisense uPAR cDNA were lysed in equal protein contents after counting cells in separate identical wells and normalizing concentrations. Lysates were separated by SDS-PAGE (10% gel) under nonreducing conditions and immunoblotted with anti-uPAR antibody. *B*, visualization of antisense uPAR cDNA down-regulation of protein expression. Since antisense effects were not clearly visualized by Western blotting, normal and antisense uPAR-expressing DU-145 cells were grown on glass coverslips and fixed with 3% paraformaldehyde. Cells were then blocked for 1 h in TBS containing 1% BSA and then probed with anti-uPAR antibody conjugated with fluorescein isothiocyanate (FITC) for at least 5 h. Slides were photographed under a fluorescent microscope.

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irrelevance of the pXf vector itself on cell phenotype as shown previously by constructing the plasmid with control cDNAs (3).

A hallmark of tumor progression is its ability to invade through the ECM into surrounding tissue. A few signaling pathways have been shown as rate-limiting for invasion. One of these is the PLCγ motility pathway, obstruction of which caused a marked decrease in cell invasion in a variety of cell lines (2, 3, 18). In our current studies, increased uPAR expression was correlated with up-regulated PLCγ activity and highly invasive cells. Therefore, we asked whether lowering uPAR protein levels in the highly invasive WT DU-145 cells reduced these cells invasiveness. To this end, we compared transmigration of WT DU-145 and WT DU-145-AS cells through Matrigel. As shown in Fig. 4, transmigration by WT DU-145-AS was drastically reduced compared to nonantisense expressing cells. These results clearly indicated that down-regulating uPAR reversed the invasive properties of up-regulated EGFR in these cells. Along the same lines, we overexpressed uPAR in the less invasive parental DU-145 cell lines (PA DU-145-SN). As shown in Fig. 4, PA DU-145-SN transmigration through Matrigel was dramatically increased compared to nonsense uPAR-expressing cells; uPAR expression compensated for the relatively less PLCγ signaling in these cells. Again, these results indicate that uPAR expression levels are both required for and contribute to invasion and thus confirm a rate-limiting role for uPAR functions.

uPAR Down-regulation Reduces Tumor Formation and Invasiveness in Mice. Our data so far showed a clear role for uPAR in tumor cell invasion through Matrigel. In our previous studies, we showed that DU-145 invasiveness correlated with

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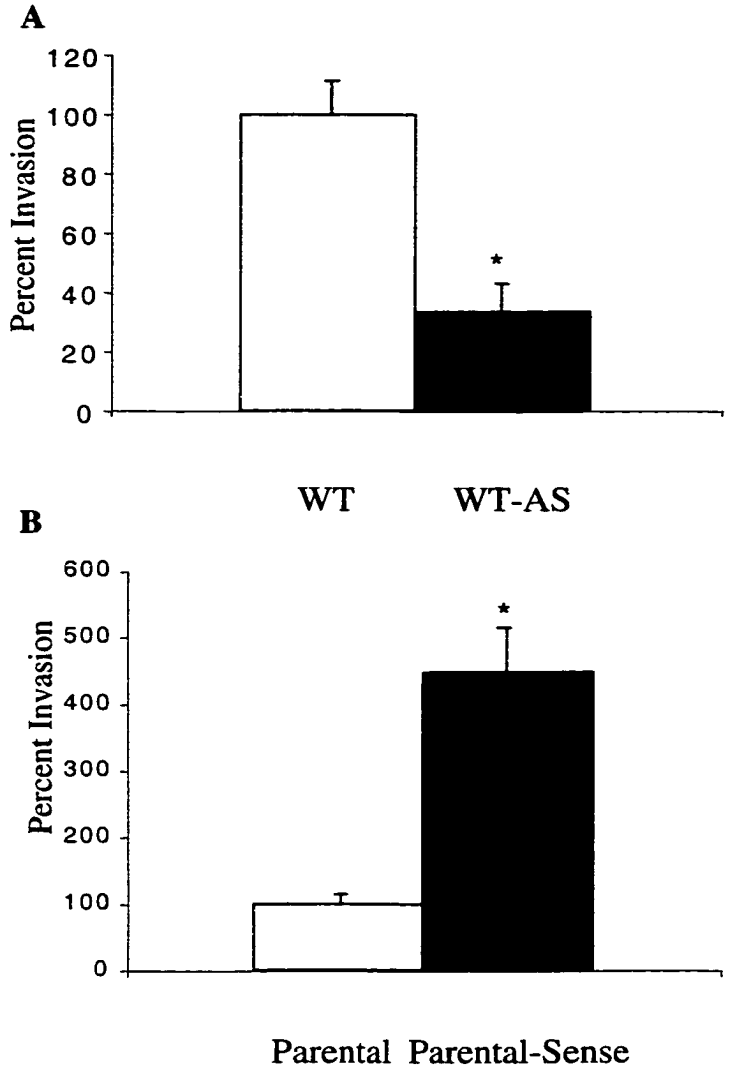

Fig. 4 Invasion of DU-145 cells is influenced by uPAR expression. *A*, to test effect of antisense uPAR on invasion of highly invasive WT DU-145 cells, 10,000 cells of antisense-expressing cells were plated into Matrigel-coated filters in transwell chambers (Biocoat/Becton Dickinson; catalog #40480) and compared to WT cells not expressing the construct. The top chamber contained media with 1% BSA for the first 24 h and was replaced with serum-free media for the remaining 24 h. The bottom chamber contained complete media with 10% FCS. After 48 h the top uninvaded cells were removed with a cotton swab, and the filter was then stained with crystal violet. Cells were quantitated by manual counting under a microscope. ■, WT DU-145 cells expressing antisense uPAR cDNA; □, WT DU-145 cells not expressing uPAR constructs, *B*, moderately invasive parental DU-145 cells were compared to the same cells expressing full-length sense uPAR cDNA for migration through Matrigel as described. ■, DU-145 parental cells expressing sense uPAR cDNA; □, WT DU-145 cells not expressing uPAR constructs; . *, $P < 0.05$

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PLCγ activation via overexpressed EGFR. Inhibition of PLCγ pharmacologically with U73122 (16) or molecularly via the dominant negative fragment PLCz (3) significantly reduced invasion of tumors formed by WT DU-145 in athymic nude mice. Because our recent data showed a clear link between enhanced invasiveness and uPAR expression, we asked whether down-regulation of uPAR expression would negate the effects of up-regulated EGFR and PLCγ activity. Being able to demonstrate such an effect in mice would be of paramount importance due to the fact that the complex interactions of the uPA/uPAR system with various integrins, growth factor receptors, and extracellular proteases can only be fully realized in an *in vivo* environment as opposed to the controlled Matrigel. We injected both WT DU-145 and WT DU-145-AS cells intraperitoneally into athymic nude mice (~2 million cells each) and allowed tumors to form over a 60-day period. As shown in Fig. 5, WT DU-145 cells aggressively invaded through the diaphragm of these mice as expected (16). However, WT DU-145-AS cells formed markedly less aggressive tumors in these mice. Although surprising, such results have been shown in another system where glioma cells expressing adenovirus-driven antisense uPAR failed to form tumors in nude mice (44). These results demonstrate a critical rate limiting role for uPAR in tumor growth, which is invariably a precursor for invasion and metastasis.

DISCUSSION

The signals promoting tumor cell growth, migration, and metastasis are intertwined in a complex medley of growth factors, growth factor receptors, integrins, ECM components, and the numerous signaling pathways that each of these induce (45). In this

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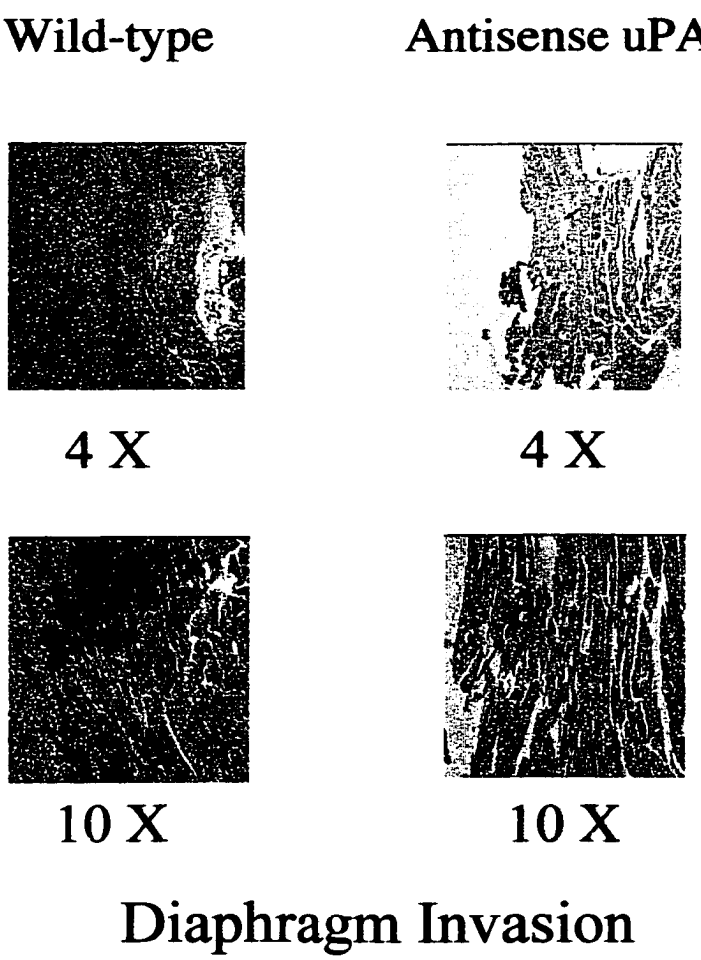

Fig. 5 Diaphragm invasion of WT DU-145 cells in athymic nude mice. Mice were injected with 2 million WT DU-145 or WT DU-145 antisense uPAR cells into the peritoneal cavity. Mice were asphyxiated after 45 days, and organ/tumor growth and metastasis were determined. Organs, including the diaphragm, were removed, fixed in 10% formalin, and stained with H&E. The left column shows a representative diaphragm taken from a mouse injected with WT DU-145, whereas the right column indicates diaphragms taken from mice injected with WT DU-145 antisense uPAR cells. *Top*, magnification at 4X; *bottom*, magnification at 10X.

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study, we probed downstream of our previously established finding that growth factor-induced migration, via PLCγ signaling, is rate-limiting in tumor cell invasion (46). While PLCγ contributes to motility induced by numerous growth factor receptors (13-15) via an epigenetic hydrolysis of PIP_2 (47, 48), there is evidence that signaling through this effector alters the gene expression profile (24). Thus, we asked whether these changes in transcription contribute directly to invasion. Using mRNA microarray analysis, we discovered a number of genes that were up-regulated in the human prostate carcinoma DU-145 cell line that was engineered to be highly invasive through enhanced EGFR signaling (WT DU-145). As the uPAR receptor surfaced as the highest elevated transcript, we focused on clarifying the role of this receptor in tumor cell progression. Using both *in vitro* and *in vivo* models of tumor invasion, we demonstrated that uPAR contributes to invasion of these prostate tumor cells and would be an extracellularly accessible target for intervention.

The fact that uPAR presented as the most up-regulated gene in a PLCγ/motility pathway enhanced system was intriguing due to uPAR's role in cell migration. uPAR is known to localize at the leading edge of the cell during the migration process (49) and has been shown to be required for migration of a number of cells (50, 51). It also is vital for promoting integrin signaling (reviewed in Ref. 38), along with various integrin-linked molecules such as focal adhesion kinase (FAK) and mitogen-activated protein kinase (MAPK) (52). These intracellular effectors also are required for signaling from EGFR and other growth factor receptors (53, 54). Thus, coordinate up-regulation of uPAR in the face of an active TGFa-EGFR autocrine signaling loop, present in practically all prostate

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carcinomas (55), would serve either to reinforce these signaling pathways or broaden the biological responses contingent upon these.

Our previous studies demonstrated a rate-limiting role for PLCγ activity in tumor cell invasion. In prostate, breast and bladder carcinomas, and glioblastoma, inhibition of PLCγ significantly retarded cell invasion *in vitro* and/or *in vivo* (3, 16, 18, 56). In both breast and prostate cells, invasion through PLCγ activity was shown to be secondary to autocrine EGFR activity, since (a) a truncated EGFR which does not signal PLCγ had reduced invasion (16), and (b) treating cells with the EGFR specific kinase inhibitor PD153035 likewise retarded cell invasion through Matrigel (2). These findings strongly suggest a pivotal role for EGFR (or other growth factor receptors) promoting invasion through the PLCγ motility pathway. However, other signaling pathways likely also contribute to invasion. Previous studies have documented the role of up-regulated uPAR in invasion of various tumors, including glioblastomas and carcinomas of the bladder,and breast (25, 26, 30, 37, 57). These tumors are the same types in which up-regulated EGFR signaling strongly correlates with invasion and tumor progression (58-60). Our results herein strongly suggest a causal link between EGFR-PLCγ signaling and uPAR up-regulation. Thus, it appears that these two surface receptors are coordinately up-regulated during progression of human tumors.

We found that uPAR function was rate-limiting for invasion. *In vitro* transmigration of the ECM Matrigel was modulated by uPAR levels. Tumor establishment and progression in mice were similarly affected by uPAR expression. Abrogating uPAR by antisense (WT DU-145-AS) reduced tumor aggressiveness in 15 mice challenged over three independent experiments, whereas all mice simultaneously inoculated with WT

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DU-145 cells presented highly invasive tumors. This finding strongly suggests that up-regulation of uPAR is a major outcome of this autocrine signaling pathway. This has obvious implications for rational therapeutic interventions in that uPAR, being on the cell surface, is targetable by noncell permeant agents. Still, for this to approach reality, the operative mechanism of uPAR signaling (i.e., protease, complexing with integrins, or direct receptor signaling) in this context needs be determined.

One last point warrants further emphasis. While uPAR gene expression levels correlated most strongly with invasiveness in our three DU-145 cells lines (Table 1), a number of other prominent receptors or ligands were also overexpressed in WT DU-145, including HGF, IGF-1, and the integrin subunit αv. Not only have these been implicated in promoting tumor cell invasion (61-64) but they are also known to interact with uPAR in promoting cell growth as well as migration and invasion (65-67). This is particularly relevant for HGF, which is activated from its pro-form by uPA-mediated cleavage (27, 68). Thus, this initial study may only be the tip of the iceberg with a panoply of genes being expressed in concert that further reinforce the motility, scatter, and proliferative properties needed for successful access to, and growth in, ectopic sites that are the essence of metastasis.

ACKNOWLEDGMENTS

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EBV-EXPRESSING AGS GASTRIC CARCINOMA CELL SUBLINES PRESENT INCREASED MOTILITY AND INVASIVENESS

by

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Submitted to *International Journal of Cancer*

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ABSTRACT

Epstein-Barr virus (EBV) is associated with a number of cancer types, including Burkitt Lymphoma (BL) and nasopharyngeal carcinoma (NPC), and is suspected to contribute to tumorigenesis. On average, 8% of gastrointestinal carcinomas were shown to carry the virus. In order to explore whether the presence of EBV in gastric carcinoma contributes to tumor progression, we examined an *in vitro* EBV-infected gastric cancer cell line, AGS, for motility and invasiveness-related phenotypic changes. In this study, we compared three sublines of AGS cells, two of which were infected with EBV virus, while the third acted as a control. We found a marked increase of both cell motility and invasion in the EBV-infected cells. We explored the roles of signaling pathways previously implicated in carcinoma motility and invasion. Neither the epidermal growth factor receptor (EGFR) kinase inhibitor PD153035 nor the phospholipase C (PLC)γ inhibitor U73122 reduced AGS motility or transmigration of a Matrigel barrier. Likewise, the extracellular-signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) inhibitor PD089035 did not appreciably influence AGS migration and neither did the PI-3 kinase inhibitor, Wortmannin. These data suggest that EBV increases migration of AGS cells by a mechanism independent of the ERK or PLCγ signaling pathways. Instead,we found that the EBV-infected cells presented increased focal adhesion kinase (FAK) phosphorylation and decreased association with an unknown phospho-protein pp^{110}. These findings suggest a role for integrin-mediated signaling in promoting EBV-associated invasiveness.

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INTRODUCTION

The Epstein-Barr virus (EBV), an ubiquitous DNA herpesvirus latently resident in some 90% of the human population, is the causative agent of infectious mononucleosis and immunoblastomas in posttransplant patients and other immunodefective hosts. It is associated with the majority of endemic BL, and virtually 100% of NPC. It is also found in anywhere from 2-18% of gastric carcinomas (1). The near complete association of the virus with NPC was taken to suggest that the virus plays a role in the development of this tumor (2). However, no phenotypic evidence has been provided to support this contention. The same question of whether EBV presence is merely an epiphenomenon is particularly true for EBV-carrying gastric carcinomas wherein the virus is found only in a minority of the tumors. Still, it has been noted that EBV-carrying carcinomas are highly aggressive (3). Thus it is conceivable that EBV expression enhances not the tumor development phase but rather the progression phase by increasing cell invasiveness.

Tumor invasion involves active motility through a surrounding barrier and into adjacent tissue (4). Studies have implicated both growth factor and adhesion receptors in promoting the invasiveness of carcinomas (5-7). However, it is difficult to separate these two modes of invasiveness since integrins and syndecans are engaged during simple cell adhesion, and most, if not all, carcinomas present autocrine signaling through growth factor receptors (4, 8). We have recently reported that invasiveness of prostate and mammary carcinoma cell lines is driven by autocrine activation of the epidermal growth factor receptor (EGFR) (9). Further blurring the distinction, the interplay between these two classes of signaling receptors has gained the forefront (10, 11) with reports on regulation of integrin expression by epidermal growth factor (EGF) receptors (12), the re-

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quirement for EGFR signaling for integrin-mediated haptokinesis (13), and direct interactions between integrins and erbB2 (14). Thus, to determine the mechanism of increased invasiveness, downstream biochemical and biophysical events must be analyzed.

A large number of molecules have been implicated in tumor invasion. For most of these the mode of activation and the biophysical outcome remain to be clarified. However, two growth factor receptor-induced pathways have been suggested that enhance motility, whish is an important step in invasion. One, requiring ERK/MAPK activity (15), culminates in calpain activation leading to cell de-adhesion from substratum (16, 17). In concept, integrin signaling shares this biochemical/biophysical linkage (18, 19); though the calpain isoform appears to be different. Calpain-I is apparently operative in integrin-mediated de-adhesion, and the up-stream activation is likely distinct, with stretch-activated calcium channels probably being involved in integrin-mediated de-adhesion (20). The second pathway, involving phospholipase C (PLC)γ hydrolysis of phosphatidylinositol bisphosphate (PIP_2), provides a distinction between growth factor receptor and integrin signaling of motility (21). EGFR, platelet-derived growth factor receptor (PDGFR), and insulin-like growth factor-I receptor (IGF-IR) require PLCγ signaling for motility (22-24), whereas haptokinesis utilizing the β1 integrin appears to be independent of PLCγ (25). Thus, by determining the requirement for specific molecules in tumor cell invasion, one can begin to tease out the initiating causes. This in turn would provide suggestions for therapeutic interventions.

A second avenue associated with cell migration and tumor invasion is the integrin cluster signaling. One key molecule involved in integrin-mediated signaling is FAK. Inhibiting FAK expression has been shown to retard cell invasion and redistribute the

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actin cytoskeleton to characterize a rounded dormant cell, and re-expression of FAK in FAK-negative cells restored their motile phenotype (26). In addition, fibroblast adhesion occurs in conjunction to association of FAK with signaling molecules such as Grb2 and c-Src (27). FAK also interacts with growth factor receptors such as EGFR and PDGFR, serving to integrate signaling of these receptors with integrin signaling (28). Thus, involvement of FAK signaling is a hallmark of cell motility, regardless of the initiating integrin trigger.

The purpose of this paper was to examine whether EBV-carrying carcinoma cells are more invasive than their EBV negative counterparts. To define the molecular basis for this, we first probed EGFR-mediated invasion pathways because our previous studies have implicated a role for EGFR signaling through at least two different pathways in invasion of other unrelated tumor types (6, 9). In this study, we tested a gastric carcinoma cell line (AGS) into which EBV was introduced by two different methods: transfection (AGS AP-1) and infection via co-incubation with EBV-infected Akata cells (AGS NeoA). Our goal was twofold: to determine whether EBV expression in these cell sublines enhances invasion, and whether such increase is mediated by previously implicated signaling pathways. We found that the EBV carrying AGS sublines are more invasive than their original EBV-negative counterpart as determined through Matrigel. This phenotypic change did not involve the EGFR, ERK, or PLCγ signaling pathways. However, additional experiments showed an increase in FAK tyrosine phosphorylation in the EBV-infected cell lines and an abrogation of FAK association with a yet undefined protein. These data lead to speculation of EBV influencing an integrin-linked invasion-

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inducing pathway, where candidate molecules such as integrin-linked kinase (ILK), paxillin, and possibly μ-calpain may play a role.

MATERIALS AND METHODS

Cell Lines and Expression of EBV. A gastric carcinoma cell line, AGS (29), was used for EBV infection. AGS is derived from signet ring cell carcinoma. Previously, EBV-infected AGS lines that have been generated by using neomycin-resistent (Neo^r) gene tagged-EBV have been described (30). We also used the Neo^r EBV (31). We infected and established two different clones of AGS cells, AGS-NeoA and AGS-AP-1, by two different methods.

AGS NeoA clone was obtained by cell-free-virus infection, as described elsewhere (32). Virus production was induced as described (30). Five million cells from the EBV-positive BL line, Akata, were suspended in 5 ml medium containing 0.5% (vol/vol) rabbit anti-human immunoglobulin G (IgG) (Southern Biotechnology), incubated for 2 h at 37°C, washed, resuspended, and incubated in RPMI supplemented with 10% FCS for 48 h. The culture medium was harvested, filtered through a 0.4 μm-pore-size membrane, and stored at 4° until use. Two million AGS cells that were EDTA-treated (1 mM for 5 min) were suspended in 2 ml diluted culture supernatant from recombinant EBV-infected Akata cells. After 2 h incubation with gentle shaking, the cells were washed and resuspended in fresh medium and incubated for 2 days. Infected cells were selected in medium containing 500 μg/ml G418. Cells were then plated in 96-well, flat-bottom plates at 10,000 cells per well with complete medium containing G418. Cells were fed every 7 days until colonies emerged (3 weeks).

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The AGS AP-1 clone was obtained by so called "apoptotic transfer" of EBV (33). Neo^r EBV-carrying Akata cells were irradiated (8,000 rad) and cultivated with AGS cells for 2 days. The cells were then EDTA-treated and EBV infected cells were plated in 96-well plates at 10,000 cells per well with complete medium containing G418 (500μg/ml).

Expression of EBV gene products in the two infected clones was confirmed by immunofluorescence. Mouse monoclonal antibodies against EBNA2 [pe-2, 1:2, (Ref. 34)] and LMP1 [CS1-4, 1:30 (Dakopatts Inc.)] were used as primary antibodies. EBNA was detected by anticomplement immunofluorescence (ACIF), using the serum of a seropositive human donor. EBNA1 was also stained by ACIF with human serum absorbed with EBNA1-deficient cell, E95-A-BL28 (35). It was confirmed that the G418-selected AGS AP-1 and AGS Neo A cells were virtually 100% positive for EBNA (Fig. 1). Further, both clones were positive for EBNA1 but negative for EBNA2 or LMP1. RT-PCR analysis for the EBNA promoter (32) revealed that both clones utilized Q promoter but no C/W promoter. Our data suggested that viral expression in the two infected AGS clones used the latency I program (36).

Matrigel Invasion Assays. Cell invasiveness *in vitro* was determined by the ability to transmigrate a layer of the extracellular matrix (ECM) Matrigel in a modified Boyden chamber assay. Matrigel invasion chamber plates were obtained from Becton Dickinson/Biocoat (catalog # 40480). For each individual cell line, cells were plated randomly and distributed among plates with different lot numbers, with each experiment performed in triplicate. Due to variation among lots due to Matrigel thickness and tex-

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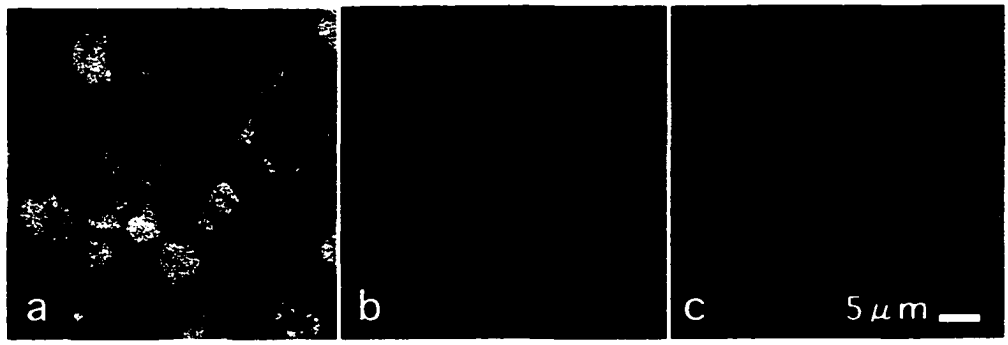
AGS NeoA Cells



EBNA1 EBNA2 LMP1

Fig. 1 AGS sublines present the latency I program of EBV expression. Immuunofluorescence confirmed that the G418-selected AGS AP-1 and AGS Neo A cells (NeoA shown here) were virtually 100% positive for EBNA1. Further, both clones were negative for EBNA2 or LMP1. RT-PCR analysis for the EBNA promoter (32) revealed that both clones utilized Q promoter but no C/W promoter (data not shown).

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ture, each lot was validated with the appropriate controls. Despite possible variances in EGFR ligand concentrations in Matrigel, these concentrations are biologically saturating (37) since even "reduced growth factor Matrigel" contains relatively high amounts of EGF (up to 0.5 ng/ml compared to 0.5-1.3 ng/ml in regular matrigel) (Becton Dickinson Labware 1997/98 catalog description, page 128). Cells were kept in serum-free media containing 1% BSA for the first 24 h and then replaced with only serum-free media for the remaining 48 h. Enumeration of the cells that invaded through the matrix over a 72 h period was accomplished by visually counting cells on the bottom of the filter, as per routine procedures, after any uninvaded cells were removed from the top of the filter with a cotton swab. In all cases, individual experiments were performed in duplicate chambers.

Migration Assays. Cell migration was determined by the *in vitro* wound-healing method (38). Cells were grown to confluence in 6-well plates and then placed in media containing 1% dialyzed FCS for 24 h (only if treated with EGF). The monolayer was then "wounded" with a rubber policeman. After wounding, cells were placed in 1% dialyzed fetal calf serum (dFCS) media containing the appropriate treatment. Using a microscope, the wound area was photographed initially and after 24 h. The distance that cells had traveled into the denuded area was determined using computer-driven image analyses, at three distinct points in the wound, and averaged. Cells were in quiescence media during the 24 h after wounding. For each wound, three individual measurements of the gap were taken. All experiments were performed in either duplicate or triplicate wells.

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Immunoprecipitation/Immunoblotting. Protein expression and phosphorylation were determined by standard procedures. Cells were quiesced for 6 h in 1% dFCS followed by stimulation with EGF for 5 min. Cells were washed with PBS and lysed in lysis buffer [10% glycerol, 1% Triton X-100, 100nM NaCl, 20 mM HEPES (pH 7.4), and 1 mM sodium vanadate] for 1 h at 4°C. After clarification by microcentrifugation, the lysate was incubated for 1.5 h with a specific antibody-agarose bead mixture that had been incubated overnight at 4°C. Primary antibodies included antihuman EGFR (Oncogene Science; catalog #GR01), anti-PLCγ antibody (Upstate Biotechnologies; catalog #05-163), anti-alpha-v integrin (Santa Cruz Biotechnology; catalog #sc-6616), anti-alpha-6 integrin (Santa Cruz Biotechnologies; catalog #sc-6597), anti FAK (Transduction Laboratories; catalog #F15020), antipaxillin (Transduction Laboratories, catalog #P13520), and anti-ILK (Upstate Biotechnologies; catalog #06-592). The lysate-antibody-agarose bead mixture was then washed five times with lysis buffer. Alternatively, cells were directly lysed in hot Laemmli buffer. Protein and phosphorylation levels were evaluated by reducing SDS-PAGE and immunoblotting.

RESULTS

EBV-Infected Cell Sublines Present Greater Invasiveness. Tumor invasion requires breaching a barrier matrix and moving into the surrounding tissue. This is best approximated *in vitro* by determining the transmigration of a biologically active matrix such as Matrigel. Cells were placed on top of the Matrigel cushion and the relative number that transmigrated to the bottom side of the porous support wereenumerated. The EBV-expressing AP-1 and NeoA sublines invaded through the barrier to a significantly

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greater extent than the parental line (Fig. 2). These data provide direct evidence that EBV-expression enhances invasiveness.

EBV-Infected Cells Exhibit Higher Migration. Active cell motility is one rate-limiting step of tumor cell invasion (4, 6). To determine whether this might be the critical component increased in EBV-enhanced invasion, we subjected all three AGS sublines to migration assays, first in the absence of any exogenous agent. Both EBV-infected sublines migrated to a significantly greater extent than the mock-infected, with the AP-1 and NeoA strains migrating ~120% and ~40% more, respectively (Fig. 3).

AGS Cells Express Non-Activated EGFR and PLCγ. Our previous studies revealed that increased motility and invasiveness of prostate, breast, and bladder carcinoma cell lines were due to autocrine EGFR signaling involving PLCγ activation (5, 6, 9). Such EGFR autocrine signaling is potentially present in most carcinomas. Therefore, we asked whether this carcinoma line was similarly driven to invade. AGS AP-1 and AGS NeoA, along with the mock-transfected sample (AGS mock), were examined for presence of these signaling molecules; all three cell lines showed similarly high expression of EGFR and PLCγ by specific immunoblotting (Fig. 4*A*). However, it was of greater importance to test whether those two molecules were activated in these cells presumably by autocrine signaling and if the extent of such phosphorylation was correlated with EBV expression. All three strains of AGS cells were subjected to immunoprecipitation analysis. Surprisingly, there was no detectable phosphorylation of either EGFR or PLCγ (Fig. 4*B*). This suggested that there is little to no inherent PLCγ activity in AGS

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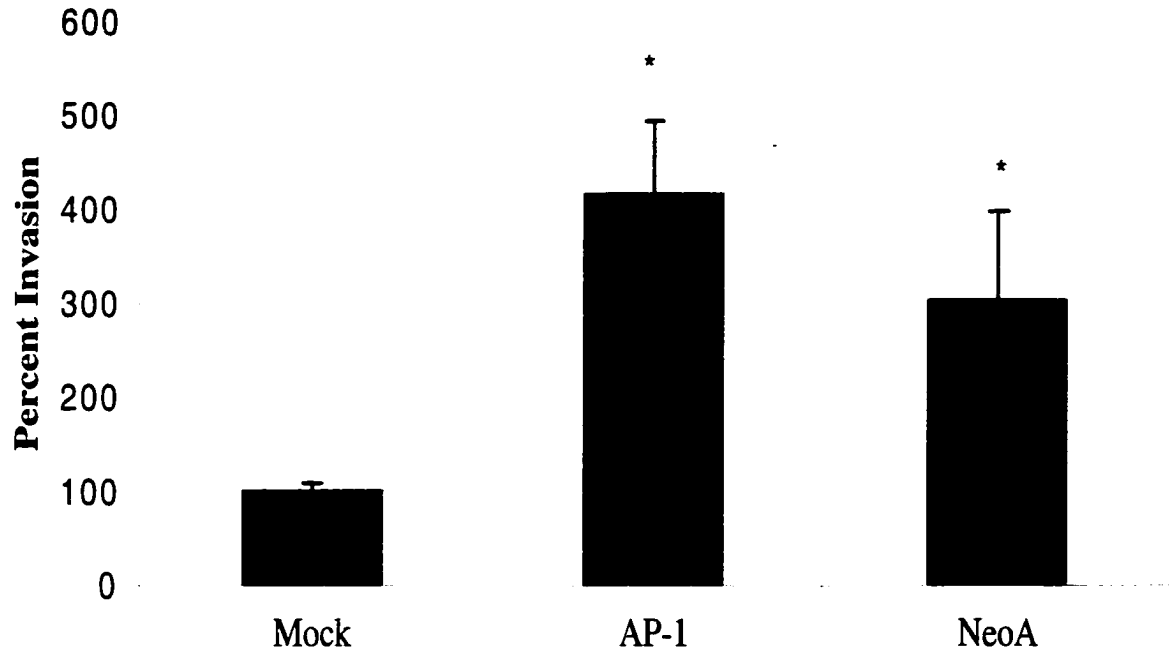

Fig. 2 EBV-expressing cells present increased invasion through Matrigel. Cells were evaluated in their ability to transmigrate through a layer of Matrigel across a gradient over a 72 h time period. Experiments were performed using modified Boyden chambers in 24-well plate configurations. The figure demonstrates a significant increase of invasiveness in both AP-1 and NeoA cells over mock-transfected cells ($P < 0.05$ for both EBV-carrying sublines). The number of cells that transmigrated through the Matrigel were normalized to mock-infected cells within each experiment. Shown are percentage invasions ± SE of at least four experiments for each cell subline, each performed in duplicate.

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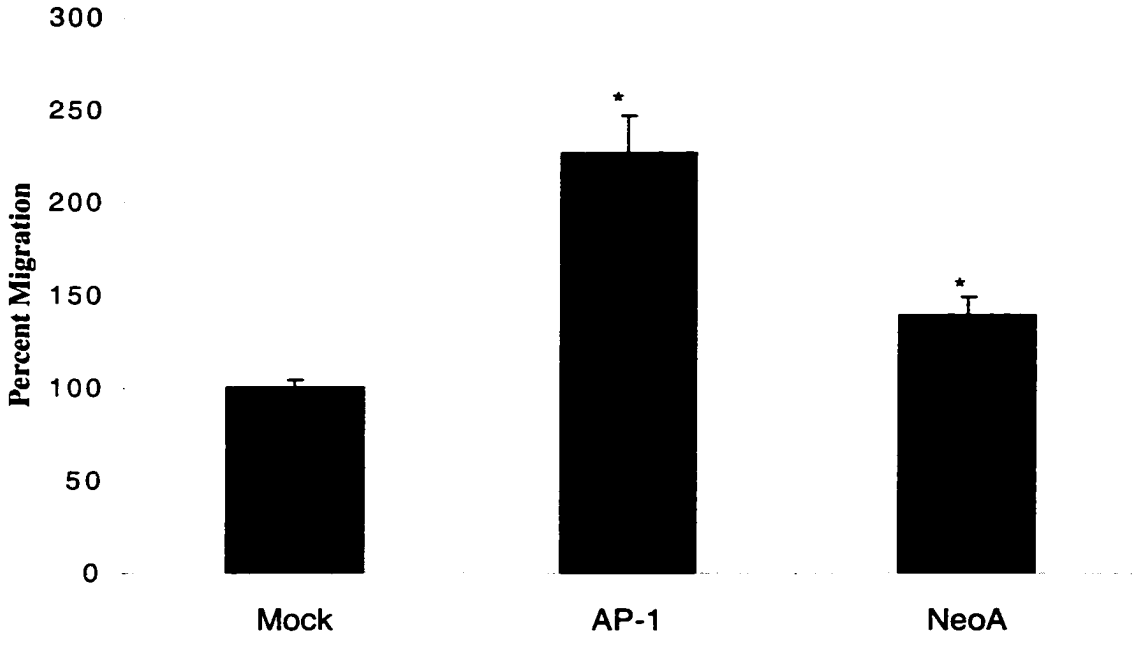

Fig. 3 EBV-expressing cells present increased migration in wound-healing assays. A portion of monolayer of cells in a 6-well tissue culture plate was denuded with a rubber policeman, after which the rate at which the created gap healed was measured with computer-assisted microscopy at 0 and 24 h after wounding. Cells were plated in serum-free media containing 0.5 μg/ml Mitomycin-C for the duration of the experiment to prevent proliferation. $P < 0.05$ for either EBV-carrying subline compared to mock-infected AGS cells. Shown are percentages of motility ± SE of at least three experiments for each subline, each performed in duplicate.

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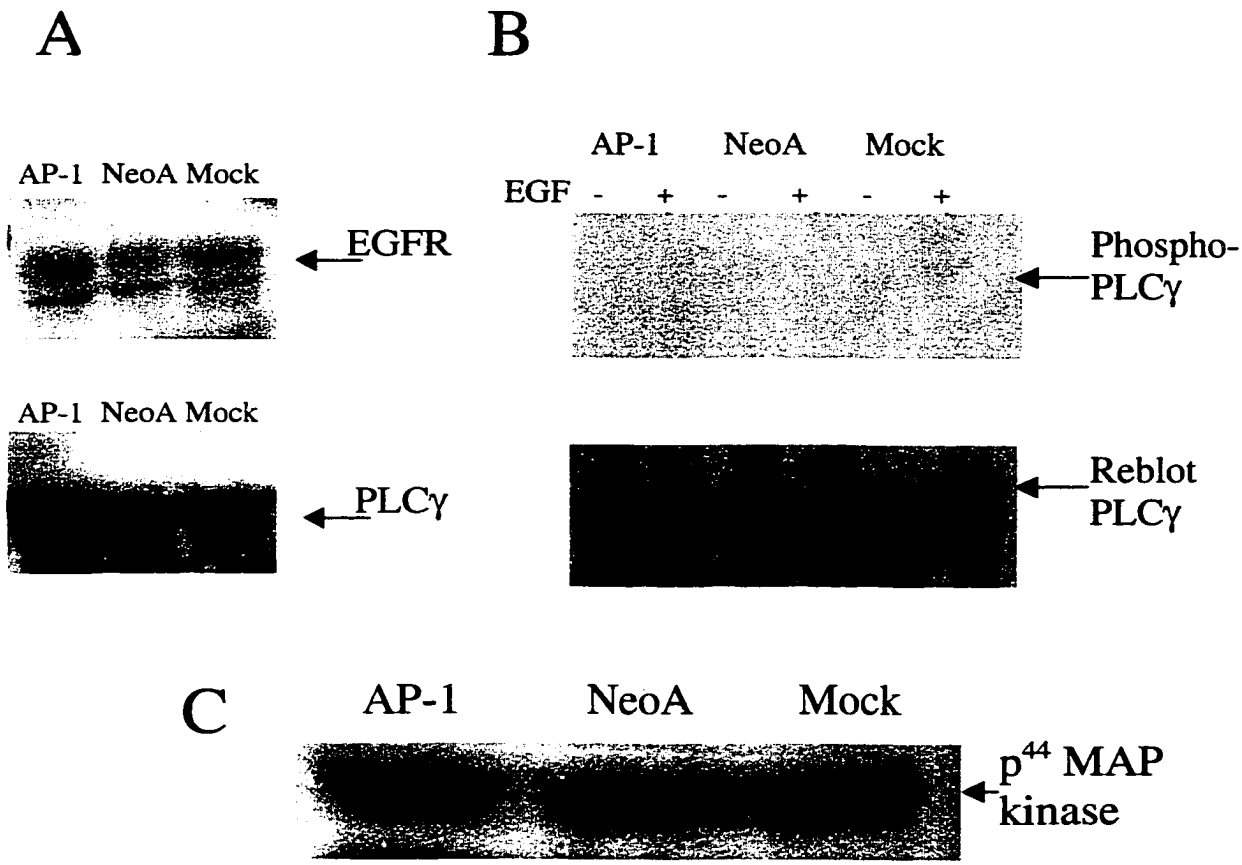

Fig. 4 The EGFR-PLCγ signaling pathway is not active in AGS sublines. *A*, EGFR and PLCγ immunoblotting. Lysates of cells were immunoprecipitated with anti-EGFR antibody (Oncogene Science; catalog #GR01) or with anti-PLCγ antibody (Upstate Biotechnology; catalog #05-163), size-fractionated by SDS-PAGE, and then immunoblotted with the same anti-EGFR antibody or anti-PLCγ antibodies. Shown are representative immunoblots of at least two experiments each. *B*, *top*: PLCγ phosphorylation. Lysates of cells were immunoprecipitated with anti-PLCγ antibody, size-fractionated by SDS-PAGE, and then immunoblotted with antiphosphotyrosine antibody (Transduction Laboratories, catalog #P11120). *Bottom*, the same gel was re-blotted with anti-PLCγ to visualize the actual protein on the gel. Shown are representative immunoblots of at least two experiments each. *C*, MAPK immunoblotting: Cell lysates were directly separated on SDS-PAGE and then immunoblotted with anti-phospho-MAPK antibody (New England Biolabs). Shown is a representative of at least three experiments.

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cells. To test whether other EGFR-promoted pathways were differentially induced in these cells, all three strains of AGS cells were probed for ERK activation as an activator of calpain-mediated de-adhesion (15, 16). ERK/MAPK phosphorylation was detectable (Fig. 4*C*), but with no discernible difference between EBV-infected and mock-infected cells.

EBV-Infected Cells Exhibit Higher Migration Through a PLCγ-Independent Mechanism. Despite this negative evidence, we asked whether EGFR and PLCγ signaling still might be contributing to motility with the reasoning that tightly coupled autocrine signaling may result in seemingly negative EGFR and PLCγ phosphorylation due to rapid down-regulation of activated molecules (39). First we tested whether EGF would enhance cell motility. EGF exposure during the "wound healing" assay enhanced the motility of all three cell lines only minimally (Fig. 5*A*). This small increase in motility might suggest an attenuated EGFR motility signal or that this particular pathway is already at least partly functioning. Thus, we targeted two key elements of this potential motility pathway. The same cells were treated with the EGFR kinase inhibitor PD153035; this abrogated the relatively small EGF-induced migration but did not reduce migration to below pre-EGF treatment levels. As other growth factor receptor motility signals also converge at PLCγ activation (23, 24), we evaluated whether the observed increase in migration correlates with PLCγ activation. Cells treated with the PLC inhibitor U73122 migrated similarly to untreated cells. However, U73122 did inhibit the slight increase seen in migration due to EGF but did not reduce the overall increase in motility of AP-1 and NeoA strains over the mock-transfected strain (Fig. 5*B*).

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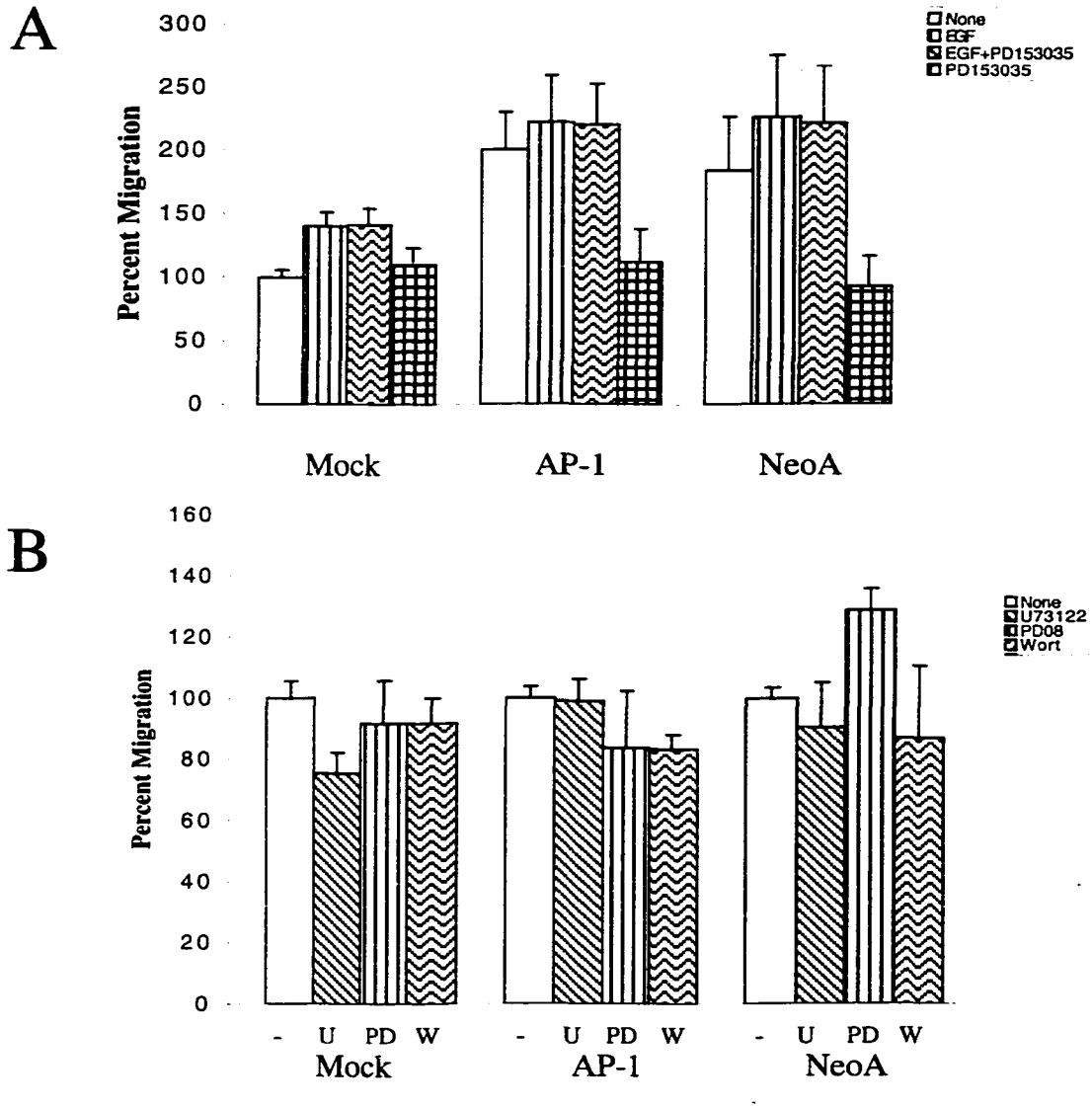

Fig. 5 Motility in EBV-infected cells is not affected by EGF. A monolayer of cells in a 6-well tissue culture plate was "wounded" with a rubber policeman, after which the rate at which the created gap had healed was measured with computer-assisted microscopy 24 h after wounding. Cells were plated in quiescence media containing 0.5 μg/ml Mitomycin-C, as well as 10 nM EGF, 500 nM PD153035, 3 μM U73122, 2 μM PD98059, or 1 μM Wortmannin where applicable, for the duration of the experiment. Shown are percentages of motility ± SE of at least four experments for each subline, each performed in triplicate. *A*, cells were treated with EGF (E) as well as the EGFR kinase inhibitor drug PD15035 (P+E) or the kinase inhibitor solely (P). *B*, as EGF stimulation had no discernible effect, cells were also treated with the PLC inhibitor U73122 (U), the MAPK inhibitor PD089059 (PD), or the PI-3 kinase inhibitor Wortmannin (Wort). None of these treatments showed any significant alterations in rate of migration of each subline

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Two other key intermediary effector molecules have been implicated in both growth factor- and integrin-mediated motility. The ERK/MAPK transmit EGF and platelet-derived growth factor (PDGF) de-adhesion (15, 40) and integrin-signaled cell contraction (41). We treated the cells with the ERK inhibitor PD098059 with no significant effect on the enhanced migratory status of AP-1 and NeoA cells (Fig. 5*B*). Phosphatidylinositol 3'-OH kinase (PI-3 kinase) has also been implicated in tumor invasion promoted by both classes of receptors (42-45). The PI-3 kinase inhibitor Wortmannin also had no effect on migration. These data suggest that any EBV-mediated activity in these cells is unlikely to be modulated by autocrine or paracrine EGFR signaling.

FAK Phosphorylation is Increased in EBV-Infected Cells. As mentioned previously, an alternate stimulator of cell migration is integrin signaling. Changes in integrin levels or localization promote invasion (46). An initial cursory exam of two previously attributable integrins, αv and α6 (46, 47), showed no difference in expression levels of these proteins (data not shown) or in cellular distribution.

There are various pathways involved due to the number of integrin receptors that could be involved; however, there are some common conversion points. In order to clarify which cellular events might play a role in increased invasion in EBV-infected cells, we examined two high profile integrin-associated conversion points, FAK (48) and ILK (49). Due to its evidently low phosphorylation levels, ILK showed no remarkable changes in levels of phosphorylation of serine/threonine (Fig. 6), while FAK tyrosine phosphorylation was elevated in both AP-1 and NeoA sublines compared to the mock-infected cells (Fig. 6). Additionally, consistent co-immunoprecipitation of a 110 kDa

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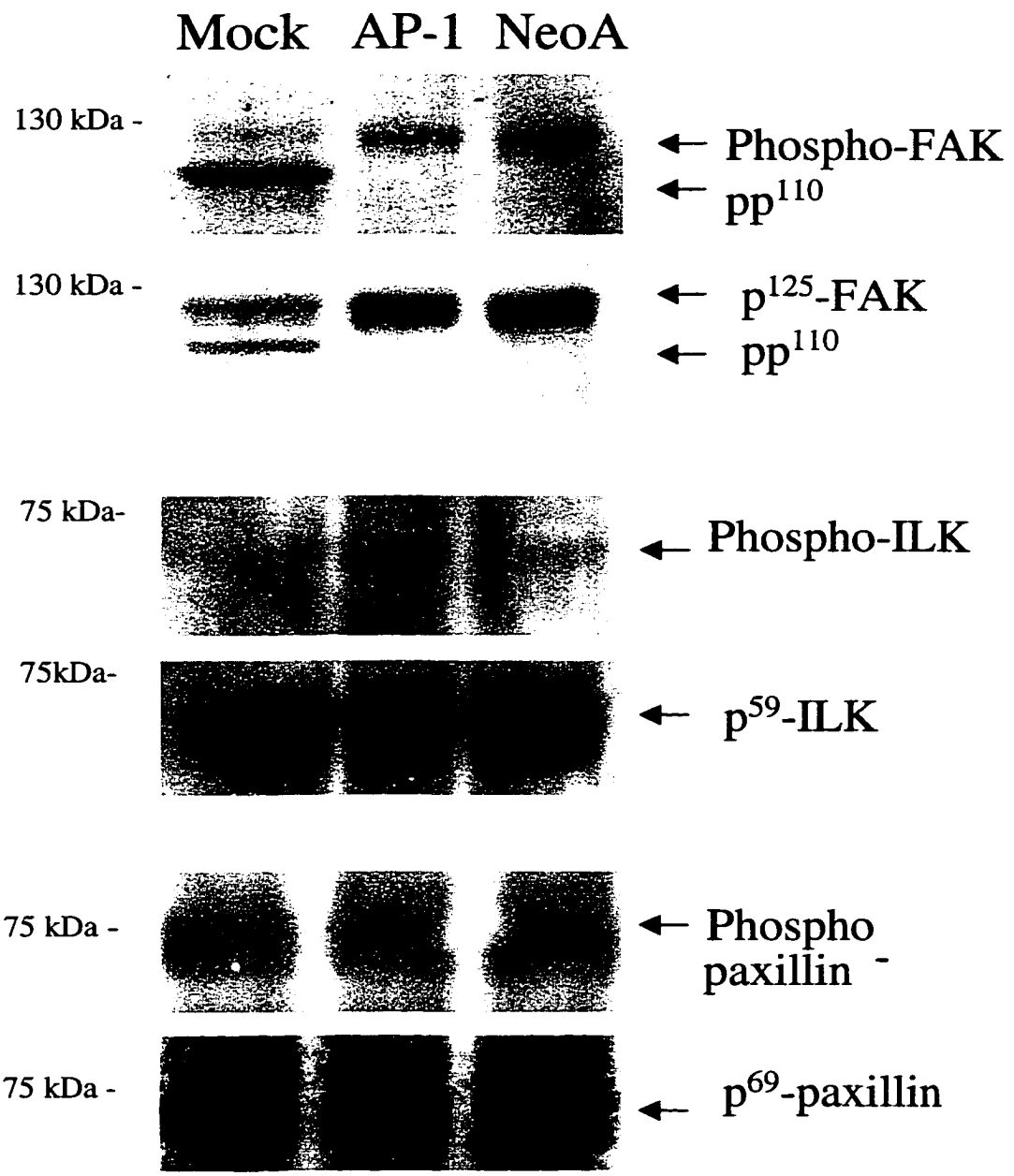

Fig. 6 FAK phosphorylation is increased in the EBV-infected AGS cell sublines. Cells were lysed in lysis buffer, and then the lysates, normalized for protein content, were immunoprecipitated with antibodies against FAK (Transduction Laboratories; catalog #F15020), ILK (Upstate Biotechnologies, catalog #06-592), and paxillin (Transduction Laboratories, catalog #13520). Proteins were separated by SDS-PAGE, transferred onto blotting film, and probed with antiphosphotyrosine antibody (Transduction Laboratories; catalog #P11120) in the case of FAK and paxillin and a cocktail of antiphosphoserine/threonine in the case of ILK. All cell lysates were subsequently re-blotted with the original immunoprecipitating antibody to visualize protein content. Shown are representative blots of three experiments.

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protein, that was detected by antiphosphotyrosine and antiphosphoserine/threonine probes (pp^{110}), with the mock-infected cells was absent in the EBV-infected strains. These data firmly point to FAK as a starting point of investigation in elucidating the events surrounding increased migration and invasion due to EBV expression.

We examined the phosphorylation of both MAPK (Fig. 4*C*) and a known FAK substrate, paxillin (50) (Fig. 6), in all sublines. In both cases, the observed differences in phosphorylation of these proteins among the three strains were not significant.

DISCUSSION

The most threatening and potentially deadly aspect of cancer is progression from a localized to a malignant invasive tumor. The molecular mechanisms responsible for such transformations are just now being elucidated. The process of tumor invasion requires the cells to attain new phenotypes that enable it to transmigrate a barrier matrix (4). One rate-limiting property is cell motility, which is controlled by a number of recently described signaling pathways (4). As recent evidence has given an example of a carcinoma-expressing EBV being more invasive (51), we tested another carcinoma cell line (AGS) where we found EBV expression correlated with increased invasion and asked if this, too, was due to increased motility and whether this occurred via previously described signaling pathways. Implication of EBV with gastric carcinoma progression would broaden the list of EBV-associated diseases and provide for new therapeutic interventions. In this study, we sought to identify adverse effects of EBV on a recently derived gastric carcinoma cell line, AGS. We found that EBV does indeed enhance the tumor progression potential of AGS cells by significantly increasing their invasion. This

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invasiveness correlated with increased motility. This finding increases the list of cell properties that might be enhanced by EBV carriage to the clinically important function of tumor cell invasion.

In order to prove that the motility process is rate-limiting for invasiveness, the biochemical mechanism underlying this increase needed to be determined. That EGFR signaling is commonly attributed to increased tumorigenicity was a reasonable assumption in this case, especially as antisense EGFR adversely affects tumor growth in parental AGS cells (52). Less uncertain, however, is involvement of EBV in the EGFR signaling pathway, which involves both PLCγ signaling for motility (9, 22) and MAPK for mitogenesis and focal adhesion disassembly (15). We were unsuccessful in our attempts to implicate these usual suspects as the driving signaling events for motility. Dissimilar to previously investigated prostate, breast, and bladder carcinomas and glioblastomas (6, 9, 53), the AGS sublines did not present autocrine-mediated motility signaling that required PLCγ activity, particularly in terms of variation between mock and EBV-infected cells. Thus, we investigated possible alternative pathways that have been implicated in receptor-mediated invasion. The ERK/MAPK pathway was presumed not to be involved due to inefficacy of its inhibitor PD089035 in reducing cell migration of the AGS AP-1 or NeoA sublines to levels similar to the mock infected cells. While ERK/MAPK activation is a downstream effect of a variety of events including integrin mediated signal transduction or Rac/Rho GTPase function (54), activity of these upstream molecules are not solely dependent on ERK/MAPK and therefore cannot be ruled out as possible mediators of EBV-induced tumor progression. PI-3 kinase was another candidate tested (45). Our studies were unable to demonstrate a role for PI-3 kinase; there was no effect on motility

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at Wortmannin concentrations of 1 μM, and higher levels proved toxic to the cells. Despite these limitations, it appears that the increased migration and invasion conferred by EBV does not utilize the molecular controls described for growth factor receptor-mediated invasiveness.

A second major class of signaling events that lead to increased invasion are those derived from integrin signals. We examined levels of integrin α subunits implicated in tumor invasion (46, 47), $\alpha 6$ and αv, with no apparent change in total levels among sublines. Initial studies of subcellular localization by immunofluorescence was similarly uninformative in our hands (data not shown). Thus, we focused on the signaling activation status of molecules downstream of most, if not all, integrin signaling, FAK and ILK (48, 49). The finding that FAK phosphorylation was increased in the invasive EBV-carrying sublines implicated this signaling pathway. However, because a second major kinase downstream from integrins, ILK, appeared not to be enhanced, it is quite possible that EBV infection directly triggers this pathway, bypassing the need for external ligation of the integrins. Downstream targets of this pathway are the focus of current studies. Paxillin appears unaffected because phosphorylation was only moderately increased as determined by antiphosphotyrosine immunoblotting. Thus, future experiments will be aimed at further dissecting the molecular outcomes of these signals and their role in the EBV-induced invasion. Of major interest was the absence of co-immunoprecipitation of a yet unidentified phosphoprotein, pp^{110}, with FAK in the EBV-infected cell lines. Attempts to identify this protein have been unsuccessful to-date as initial isolation and peptide microsequencing have yielded uninterpretable data. Possibilities include EBV playing a role in depleting pp^{110}, dissociating it from FAK, substituting for it, or some

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combination of these. Whether pp^{110} plays any role in suppressing tumorigenic properties in mock AGS cells also remains to be determined.

It is prudent to implicate virally encoded proteins in the observed increase in motility and invasion of EBV-infected cells. Some studies hint for a role of the EBV-encoded LMP1 in increased tumorigenicity of EBV-expressing cancers (55). However, as mentioned previously, our AGS sublines did not express LMP1 or EBNA2, which incidentally have been reported not to be required for gastric epithelial cell growth (56) in distinction to the many situations where at least LMP1 is required for growth in a number of other tissue types. Furthermore, AGS and other gastric carcinoma cell lines are suggested to contain an alternate undefined virus receptor that is independent of the more commonly studied CD21 (30). These data indicate a rather complex and nonmainstream series of events that follow EBV infection of gastric carcinoma cells, including the AGS line used in our experiments, which will undoubtedly set further studies apart from conventional observations of the role of EBV in other tissue types.

Nevertheless, our studies have provided a starting point of investigation of a possible point of convergence of signaling of virally-encoded proteins and endogenous cellular mechanisms. This convergence point is FAK, which is up-regulated in EBV-infected AGS cells. It remains to be determined whether FAK phosphorylation is caused by direct stimulation of a virally encoded protein or, much more likely, from a yet undefined cellular stimulus (i.e., integrin clustering), which in turn is mediated by EBV-encoded proteins. Also possible are EBV-induced transcriptional alterations, which in turn propagate to induce the observed phenotype of increased motility and invasion. Although these cells lack the LMP1 oncogene, other gastric tissue-specific alternatives

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have been proposed, such as BARF1 (57). The emergence of such candidate viral oncogenes will undoubtedly shed more light on the mechanisms of EBV-influenced tumorigenic potential and their relationship with the endogenous cellular signaling mechanisms.

In summary, we demonstrated increased invasion and migration *in vitro* in the EBV-carrying sublines. Previously described receptor-mediated signaling pathways that lead to increased invasiveness appear not to be critical for this property in these gastric carcinoma cells, though integrin signaling might be involved. However a third possibility exists. EBV expression in AGS cells may alter the transcription profile and thereby enhance motility. In fact, data point to motility and invasion requiring *de novo* transcription (38, 58, 59). Thus, it is possible that EBV bypasses upstream signals and directly alters the cell's proteome to enhance cell motility. Further studies will explore whether EBV-enhanced invasion and motility is effected through transcriptional changes and whether the involved genes also are required for growth factor- and integrin-mediated invasion.

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SUMMARY

Tumor cell invasion is a turning point in cancer progression, as invaded tumors present significantly worse patient prognoses and decreased survival. The molecular mechanisms of invasion are a collaborative progression involving the coordination of multiple cellular processes. Dissecting these mechanisms to attain a better understanding of invasion and to define targets for therapeutic interventions is of paramount importance. The evidence presented in this dissertation strongly indicates that the process of cell migration as a whole is a crucial component of cell invasion. The studies presented here vastly expand on earlier preliminary work that described a proof of concept for this claim and establish a universality of this concept regardless of tissue type or mode of motility dysregulation. Furthermore, the studies reported here provide firm evidence that the cellular machinery involved in migration may be a concerted collaboration of multiple processes that collectively "shift" to a dysregulated phenotype upon stimulation or induction of only a few precursor pathways by signaling receptors. It is plausible therefore that motility is an overall phenotype of multiple pathways, all of which play an essential role in modulating distinct stages of migration and are triggered by specific initiator molecules.

PLCγ is Vital for Tumor Cell Invasion. The concept of abnormal cell migration being an integral part of invasion has gained recognition over the past several years, with more studies aimed at pinpointing the intricacies and foundations of these abnormalities

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(5). Invasion in itself is a single component, although a vital one, of tumor progression, as it can be blocked without affecting tumor growth (188). Preliminary work that preceded these studies had targeted a specific motility-signaling pathway that had identified EGFR stimulation of PLCγ as central for stimulated cell motility. EGFR was regarded as our molecule of focus due to its unambiguity regarding stimulating ligands (125) as well as it being a well-defined representative receptor tyrosine kinase with regard to other receptors such as PDGFR and insulin-like growth factor-I receptor (IGF-IR) (6). EGFR is also commonly overexpressed in a variety of tumor types (7-14). The first objective of this study, therefore, was to determine whether blocking cell motility, via inhibition of PLCγ, would retard invasion of tumor cells. We chose cells from aggressive tumors such as prostate, breast, and bladder, all of which express high EGFR signaling in a significant portion of *de novo* human tumors (8-10). Inhibition of PLCγ in these cells delayed invasion *in vitro* (manuscript 1, Fig. 4) or *in vivo* (manuscript 1 supplement, Fig. 2). These results are consistent with the importance of PLCγ in cell invasion, especially as the methods employed to inhibit this molecule were targeted and specific (manuscripts 1 and 2). The importance of PLCγ-mediated mechanisms in tumor cell invasion is also supported by the fact that it is likely to be a convergence molecule for multiple RPTKs. EGFR, PDGFR, IGF-I receptor, and ErbB2 have all been previously shown to activate PLCγ (15, 28, 189, 190). Furthermore, these receptors have all been shown to be upregulated in tumor tissues.

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PLCγ Activity is Secondary to EGF Stimulation. A central tenet of our studies and those of others (191-193) is that autocrine signaling promotes tumor progression. Therefore, we tested whether PLCγ activation was secondary to EGFR autocrine signaling. In our studies with prostate (TRAMP, manuscript 1), breast (MDA-468, manuscript 1), and bladder (253J-B-V, manuscript 2) cells, inhibition of EGFR kinase activity abrogated PLCγ phosphorylation and also reduced invasion of these cells through Matrigel. These observations demonstrated the dependence of PLCγ activity on EGFR stimulation. However, the dramatic inhibitory effect of the EGFR kinase inhibitor PD153035 on PLCγ phosphorylation is attributed to the fact that these cells have EGFR as the dominant growth factor receptor. Patient tumors might overexpress other PLCγ-activating receptors, including PDGFR or ErbB2. In our own studies with MDA-361 cells that have high expression of ErbB2 but not EGFR, PLCγ was active in the absence of EGF (due to constitutive activity of ErbB2). Moreover, inhibition of PLCγ delays cell invasion through Matrigel (manuscript 1). This provides an example of PLCγ activity being modulated by another RPTK. PLCγ therefore is likely to be a point of convergence for many such RPTKs.

It is important to note that inhibition of EGFR activity might have multiple effects (194). Therefore, it would be erroneous to conclude that the inhibitory effect of PD153035 on EGFR activity is solely due to its inactivation of PLCγ phosphorylation sites. For example, an obvious consequence of inhibiting EGFR kinase activity is inhibition of other SH2 domain-containing molecules that also are activated by EGFR. Some of these are also involved in migration, such as Src (156, 157). EGFR has also been shown to modulate migration pathways that are dependent on its kinase domain activation of the

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MAPK pathway, principally focal adhesion disassembly (17, 95). These studies have attributed EGFR inactivitation to inhibition of MAPK-dependent focal adhesion disassembly, thus abrogating the final step of migration, which is detachment (18). This is supported in our own studies in which inhibition of calpain, which is required for rear detachment, resulted in inhibition of invasion of bladder cells (manuscript 2). This illustrated the equal requirement of a second independent arm of RPTK-mediated migration in tumor cell invasion.

EGFR activity also appears to influence separate cell migration mechanisms. There are a number of studies to illustrate this. First, as mentioned in the introduction, there are various avenues of interaction between EGFR on one hand and integrins and other focal adhesion components on the other. Molecules that mediate such interactions may include c-met, uPAR, and ILK (103, 137, 195). Each of these molecules has been shown to exhibit some function that requires concurrent EGFR activity, although the cross-activation pathways are still not fully lucid. Second, EGFR contains binding sites to actin as well as postulated interaction sites with ECM components such as tenascin, which contain EGF-like repeats (149, 196). Indeed, in at least some cells, invasion requires maintenance of the integrity of the actin-binding domain of EGFR (197). Therefore, it is likely that EGFR and possibly other RPTKs participate at some level in actin cytoskeletal rearrangement to accommodate the invasive phenotype. Last, there is direct correlation between EGFR activity and transcriptional changes; transcription factors, including *fos* and *jun* (198-200), are among those activated by EGFR. Our own studies have unearthed a number of genes that are differentially expressed depending on EGFR activity (discussed below).

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It is easy to construe that general receptor activation in itself is a migration or invasion event; on the contrary, receptors such as the estrogen receptor are promoters of tumor growth but not invasion. In fact, estrogen receptor (ER) suppresses invasiveness (201). Even activation of a common RPTK, PDGFRα, leads not to motility and invasion but overwhelmingly to proliferation (202). This is attributed to the fact these receptors do not activate key signaling pathways to motility, such as that through PLCγ. Interestingly, levels of EGFR and ER in tumor cells seems to correlate inversely (203), with up-regulation of the latter signifying relatively better prognoses and higher survival rates.

Migration Per Se as a Rate Limiting Step For Invasion. The goal of our initial investigations was to elucidate the role of the PLCγ motility pathway in tumor cell invasion. As PLCγ inhibition led to significant retardation of *in vitro* and *in vivo* invasion or metastasis in various cell types, this pathway was definitely determined to play an integral role in invasion and tumor progression. However, as cell migration is a multistep process in which the PLCγ pathway is merely one arm, the question arose whether inhibition of other migration-inducing pathways results in subsequent inhibition of invasion. Separate studies have elucidated focal adhesion disassembly as another arm of EGFR-modulated migration (6, 18). These studies showed that EGFR-dependent phosphorylation of MAPK is a rate-limiting step in disintegration of focal adhesions, which is essential for rear detachment of the cell during migration (95). A vital component of this process is calpain cleavage of focal adhesion components, and because calpain activity is modulated by EGFR-dependent MAPK activity and is independent of PLCγ activity (17), we targeted this molecule to ascertain whether its inhibition retards invasion. In aggres-

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sive 253J-B-V bladder carcinoma cell lines, inhibiting calpain activity significantly retarded invasion of these cells through Matrigel, an observation that followed their reduced migration *in vitro* as a result of calpain inhibition.

Inhibition of calpain may be a more potent blocker of migration, and therefore of invasion, than inhibition of PLCγ. The rationale for this is that focal adhesion disassembly is an absolute requirement for a cell to complete its spatial shift, and calpain has been shown to cleave a number of vital focal adhesion components, including FAK and talin, as well as the β subunit of integrins (204, 205). On the other hand, PLCγ signaling can be compensated for, albeit far from completely, by other actin modifying signals such as those elicited by PI-3 kinase. This theory may explain the greater reduction of invasion caused by calpain inhibition compared to that caused by PLCγ inhibition in our studies of bladder carcinoma cells (manuscript 2). Thus, invasion can be blocked by inhibiting separate biochemical steps that are required for the biophysical process of motility.

As calpain and PLCγ activities are not mutually exclusive, our evidence strongly implies that the rate-limiting step of motility in invasion is not embodied in a single migratory pathway but rather rests in the process of migration as a whole. Separate studies have corroborated this hypothesis, as manipulation of separate focal adhesion components, most notably integrins, retarded migration and also inhibited tumor cell invasion. For example, blocking α2β1 integrins impairs migration and ECM reorganization in melanoma cells; overexpression of this integrin is associated with higher invasion and metastasis (206). In our study, we demonstrated a single system in which inhibition of distinct motility-inducing pathways resulted in retardation of invasion. These results also show that, despite the independent nature of these pathways, invasion-promoting motility

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pathways originated from a common RPTK, i.e., EGFR in the case of our studies. These collective observations gave rise to a subsequent general inquiry; the question of the existence of possible linkage between the different branches of induced motility. Although various examples of such indirect linkages have been discussed in the Introduction, we aimed for a more direct approach by studying genetic variation that accompanies induced migration. This is described next.

Growth Factor-Induced Migratory Pathways May Be Linked at Multiple Levels. As described previously, a myriad of receptor signaling pathways are responsible for initiating and maintaining the motile phenotype in a cell, which is a process that can lead to invasiveness when dysregulated. However, it is yet unknown what supplementary cellular events either precede or follow the advent of such migratory dysregulation that may occur. As growth factor and other receptors are known to modulate transcription factor activity in a number of studied systems (198-200), we utilized gene microarray technology to analyze the transcriptional discrepancies in cells that were engineered to possess various degrees of motility.

We discovered a number of genes that were up-regulated in a highly motile (and highly invasive) variant of the DU-145 prostate carcinoma cell line and a separate handful of genes that were up-regulated in the least motile variants (manuscript 3). Of interest about these findings was the relevance of some of the differentially expressed gene products found to RPTK-related motility and invasion signaling. For example, HGF and IGF-I were up-regulated in wild-type EGFR-overexpressing cells. The integrin αv was also up-regulated. These findings immediately shed light on possible cross-activation events of a

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number of different invasion-promoting growth factor receptors, in which most of these receptors play a role. We focused on the highest up-regulated gene, uPAR. This protein, which was not only up-regulated in EGFR-overexpressing cells but also downregulated in cells expressing a motility signaling-impaired EGFR construct, plays a significant role in migration according to many recent studies. It is concentrated at the leading edge of a migrating cell and is thought to mediate lamellipodial extension and focal adhesion assembly (115). As this molecule was up-regulated in invasive DU-145 cells, we asked whether manipulating uPAR expression could alter the invasive phenotype. We accomplished this by constructing antisense uPAR cDNA which, when expressed in highly invasive DU-145 cells overexpressing wild-type EGFR, reduced invasiveness through Matrigel and tumor formation *in vivo*. Overexpressing sense uPAR cDNA in less invasive DU-145 parental cells increased their invasiveness. These results extended our understanding of the downstream elements and secondary cellular processes that accompany up-regulated signaling of migration through EGFR overexpression and PLCγ hyperactivity and established a causal link between a single transformation event (EGFR overexpression) and subsequent invasion-enhancing occurrences (up-regulation of uPAR and other genes).

The fact that uPAR overexpression was secondary to and mimicked the effects of EGFR overexpression and PLCγ signaling in our study is indicative of at least three different points. First, these results reinforce evidence of cross-talk between different motility-signaling molecules. As discussed previously, there are many modes of indirect signaling channels between distinct signaling pathways and their key molecules. For example, the integrin α3β1, a laminin receptor, was shown to act in concert, at least

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partially, with EGFR activity in migrating and invading glioma cells, as inhibition of either receptors reduced migration and invasion (207). Other examples of such co-dependencies include a purported dependency of EGF stimulation for α6β4 integrin migratory signaling, in a manner independent of Shc phosphorylation by EGFR (208). In our own studies, we established that EGFR signaling of PLCγ is a promoter of uPAR activity, which in turn is also a rate-limiting factor in tumor cell invasion. Incidentally, a previous study correlated up-regulated uPAR and uPA mRNA expression in glioma cells with stimulation with TGFα, an EGFR ligand (209). Our study has elaborated on this by implicating the EGFR-modulated PLCγ cell migration pathway with this phenomenon, as cells exhibiting a fully mitogenic but nonmotogenic EGFR construct showed down-regulated uPAR expression. This is significant in that it provides evidence of linkage between molecules that mediate common cellular processes, i.e., migration and invasion, and not just individual molecules or signaling pathways per se.

Second, our findings presented here highlight a new source of interactions between separate pathways mediating similar processes, gene transcription. Our evidence suggests that, apart from mediation between molecules occurring via protein interaction (e.g., EGFR-activated Src recruitment to integrins or PLCγ association with Gab1 being required for HGF-induced tubulogenesis) (210), growth factor receptor stimulation may actually also influence molecules of other migratory pathways by modulating their transcription. Recent evidence concurs with this concept; for example, the transcription factor AP-1 was shown to be required for EGFR-induced activation of migration and invasion events such as cytoskeletal rearrangement and membrane ruffling (176). AP-1 was also required for EGFR modulation of the Rho family GTPases, which are "molecular

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switches" of integrin-mediated migration and invasion. Furthermore, overexpression of the hyaluronan receptor CD44s, which is implicated in tumor cell invasion and metastasis, is also dependent on growth factor receptor-induced activation of AP-1 (211). Our studies, which tie EGFR/PLCγ activity with gene regulation of another highly active proponent of invasion, uPAR, shed light on the possibility that gene regulation by invasion-promoting RPTKs such as EGFR is a major avenue of inducing invasion signals in transformed cells.

Third, our studies with PLCγ motility pathway modulation of uPAR expression may reinforce the notion that invasion is a consequence of dysregulated migration as a whole process as opposed to individual pathway irregularity. This can be construed due to the multiple channels of invasion-promoting events that are activated due to overstimulation of growth factor receptor-mediated signaling. EGFR signaling, which in itself modulates a series of pathways that signal migration and promote invasion, is responsible for driving activation of separate signaling mechanisms through a series of methods, including common molecule phosphorylation or, as illustrated in our study, gene transcription. Of particular interest in our study was the fact that it was not EGFR per se but rather a subprocess of EGFR activity, i.e., migration signal-promoting or -inhibiting, that elicited the observed transcriptional up-regulation or down-regulation of uPAR, consecutively. Overexpressing a fully mitogenenic (MAPK-signaling) EGFR construct that was PLCγ-signaling impaired resulted in down-regulated uPAR transcript expression (212). Thus, our study strongly suggests that receptor-mediated signaling of the migratory event as a whole, through the necessary pathways, promotes a secondary cell-wide response that collectively modulates a single process, in this case motility.

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Invasion-Promoting Signaling Pathways Are Not Necessarily Codependent.

Our studies thus far have elucidated an important concept of the relevance of cellular migration as a whole process to promoting tumor invasion. Complex networks of pathways are involved in promoting migration and are often direct proponents of invasion. As a final part of the studies undertaken for this dissertation, we considered the critical role of the EGFR-stimulated pathways such as PLCγ migration and asked whether any or ail of these pathways are an absolute requirement for migration-precursored invasion. We obtained a set of cell lines that were defined by the presence or absence of an invasion-associated virus, EBV, and tested these for validity of our hypothesis presented here (manuscript 4). We discovered that, in these gastric carcinoma (AGS) cells, EBV-infected sublines were more invasive *in vitro* than EBV-free cells. The EBV-infected sublines were also found to be more motile as determined by *in vitro* wound-healing assays. We followed on these experiments by testing which migration pathways were induced differentially in these cells but surprisingly found no correlation of increased invasion with the "usual suspects" which included PLCγ, PI-3 kinase, and MAPK. In other words, inhibition of these molecules did not retard migration of these cells. The results of these experiments became more intriguing when it became evident that the only differentiating factor in the EBV-infected versus noninfected cells was an up-regulation of FAK activity. As previewed in the introduction, FAK is a critical mediator of integrin-stimulated cell migration and is a vital component of focal adhesions. Although we did not identify the integrins that were putatively modified as a result of EBV-infection, it is a valid assumption that FAK precursors are involved in alteration of its activity. Although

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the implications of these findings are intriguing, they are also insightful of the realities of cell migration signaling.

It can be derived from these findings that, while migration-promoting pathways do interact on the protein or nuclear levels, they remain for the most part independent. Although different chemokinetic migration-promoting events tend to cross-activate each other, there is no proof that the majority of migratory pathways are collectively linked as a prerequisite. On the contrary, our studies support the concept of self-sustaining activity for individual receptor-mediated migration pathways; increased FAK activation in the more migratory and invasive EBV-infected AGS cells did not correlate with increased PLCγ activity. Because recent studies support a mediated activation of PLCγ by FAK, at least in COS cells (85), it would seem evident that the mechanism of EBV-influenced FAK hyperactivity bypasses the conventional cellular machinery, especially since neither ILK or paxillin activity are altered in EBV-infected cells according to our results. These findings, which describe an outside stimulus (EBV) that activates certain migratory pathways but not others, lend support to the notion that individual migration pathways may be sufficiently self-sustaining to contribute to the migratory phenotype that is conducive of cell invasion. Other studies have also produced evidence of this. For example, the roles of both EGF and uPA in promoting tumor cell invasion are well documented (6, 195), and our studies have shown that increased uPAR transcription is secondary to EGFR/PLCγ pathway activity. However, despite the fact that one pathway enhances activity of another, separate studies have shown that their modes of action may be entirely different. In cells expressing ECM-rearranging matrix metalloproteinases (MMPs), EGFR downstream activity was found to be reliant on MMP modulation for its invasion-

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enhancing activities, whereas uPA was not (213). These data, however, do not imply that any one of these self-sustaining pathways is *sufficient* for migration; as each is necessary for modulation of a certain stage of motility, be it extension, adhesion, or de-adhesion. Most importantly, these results show that cell invasion is not necessarily dependent on single migration pathways such as the PLCγ pathway but is nevertheless greater in cells that exhibited increased motility through yet undefined pathways. This is strong evidence that cell migration, regardless of its mode of stimulation, is a definite driving force behind tumor cell invasion.

Reconstruction. The studies reported in this dissertation contribute to gaining a deeper insight into the mechanisms of tumor progression. Multiple processes are consecutively involved in tumor progression and occur in three stages: cell recognition and binding to the ECM, rearrangement of theECM, and migration through the ECM to the new locale. We have focused on the last stage and have unearthed a wealth of information leading to a better understanding of the processes and cellular functions involved in migration. In doing so, our studies provide important clues pertaining to pathogenesis and possible effective clinical interventions.

The EGF receptor is a pivotal player in cell motility, and its irregular expression or hyperactivity, as described in our studies and others', correlates with invasiveness. Indeed, the EGFR is a prime marker for tumor cell invasion and is associated with increased aggressiveness and decreased survival. EGFR mediates a plethora of signaling pathways and has common points of convergence with other growth factor receptors such as PDGFR or IGF-I receptor, as well as integrin receptors. Integrins are a vital compo-

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nent of cellular attachment points that are crucial to lamellipodial extension. EGFR stimulation and activation mirrors that of other receptors that respond to ligands in an auto-, para-, juxta-, or endo-crine fashion, which is heavily dependent on the cellular environment. Tumor cells often retain this ligand stimulation manner and, in some cases, are better exposed to ligands since ECM degradation that precedes cell migration may release "trapped" ligands that are normally not exposed to the side of the cell surface rich in receptors, thus greatly enhancing the hyperactivity of EGFR and its downstream pathways and increasing tumorigenic potential (214).

The pathways that are modulated by EGFR include those that modulate cell mitogenesis, through the Ras-MAP kinase and STAT pathways. Our studies, however, focused on the pathways that stimulate cell migration, namely PLCγ-modulated actin modifying protein release and Erk/MAPK/calpain-regulated focal adhesion disassembly that is required for rear end detachment. The studies described here have contributed to understanding the divergent and often independent signals that are secondary to EGF stimulation. Fig. 1 is a schematic of the important pathways eluded to in this dissertation, and it is immediately obvious that EGFR-mediated signals are key regulators of cellular function, including migration. While EGFR controls a myriad of pathways, it is also involved in either facilitating or indirectly allowing for signals of other receptors to function, such as linkage with integrins and their associated molecules. Our studies showed that EGFR-mediated migration modulates cell invasion both *in vivo* and *in vitro*. We showed this in a variety of breast, prostate, and bladder cell lines, while others have shown similar effects in colon carcinoma and glioblastoma, indicating that EGFR-mediated cell migration is a universal concept. Our attempts to dissect EGFR-induced motility signaling highlighted

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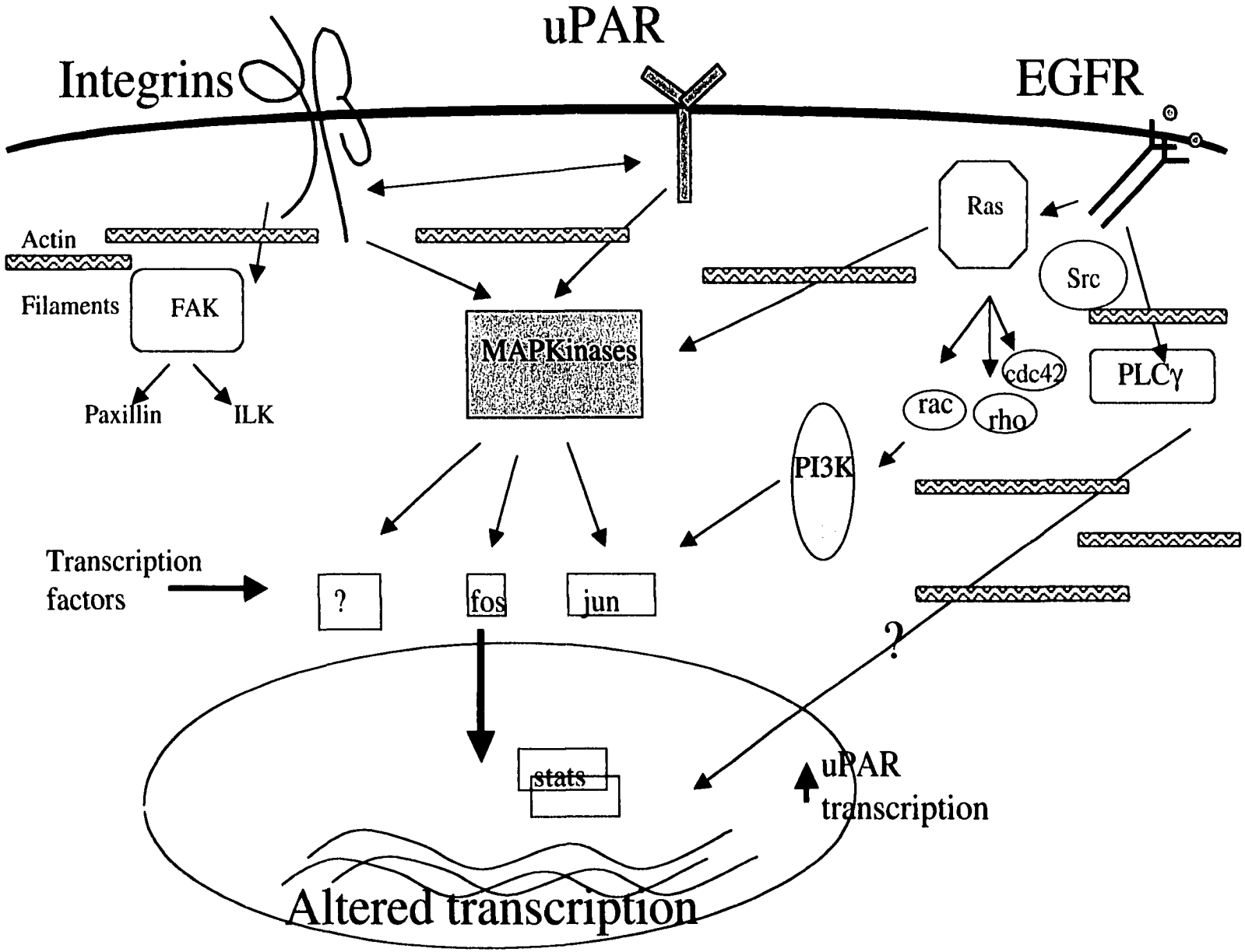

Fig. 1 Receptor-stimulated migration pathways.

two independent pathways (PLCγ-induced cytoskeletal rearrangement and calpain-mediated focal adhesion disassembly), which individually hindered cell invasion when blocked; this was evidence of tumor cell invasion being a result of EGFR (or other growth factor receptor)-mediated migration per se and not of any one particular migration pathway.

In order to define the broader consequence of stimulation of EGFR-induced migration on a cell-wide scale, we studied the differential activation of 9,216 individual candidate genes in prostate cancer cells that were engineered to exhibit differential migration and were proportionately differently invasive. These studies linked EGFR-mediated migration through the PLCγ pathway (and not mitogenesis) to up-regulated uPAR expression. uPAR itself is a heavily touted tumor promoter and is associated with integrin-dependent tumor progression. Our studies did not necessarily show a codependency of all of these pathways; on the contrary, studies with cells exhibiting up-regulated migration and invasion through nonconventional means (EBV infection) showed increased FAK activity (hence integrin and focal adhesion modulation) but not PLCγ phosphorylation. This argues that motility is a convergence point of multiple initiator signals and biochemical pathways. Regardless of mode of migratory stimulation, however, more invasive cells were consistently more motile in all our studies, signifying a cornerstone position of migration as a precursor and requirement for cell invasion.

Studies in this dissertation point to the following overall hypothetical mechanism: A transformation event, such as increased EGFR activity, consequently helps shift the cell toward a threshold above which cellular mechanisms that promote migration and, thus, invasion collectively become dysregulated. A number of earlier studies have linked

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vital signal initiators or inducers of separate processes together. This is further supported by our studies that solidified the concept of numerous genetic changes that accompany dysregulation of a single pathway, which communally, though not always interactively, enhance the invasive phenotype. This represents an intriguing leap forward in the understanding of cellular transformation events and sheds better light on topics for future studies. Indeed, future studies may not concentrate on understanding roles of individual molecules per se. Instead, they may focus on elucidating the various interactions and cross-activation of the components of individual separate tumor-promoting pathways.

Invasive and metastatic diseases are a major obstacle to cancer treatment. Therefore, studies that dissect the mechanisms involved in progression to these deadly phenotypes, such as the present dissertation, are important contributions towards development of efficacious medical interventions.

Therapeutic Implications. Because the studies presented here delineate some mechanisms of invasion through migration-promoting pathways and because these pathways work independently of each other despite cross-linkage in any instances, it is possible to select new targets for therapeutic interventions. Our studies contribute to assigning effective targets for intervention by providing for downstream convergence pathways of different migration-inducing receptors. An obvious target would be PLCγ, as it is one such convergence point of RPTKs and is a critical molecule in tumor cell invasion due to its motility-signaling pathway. Such a target would be more prudent to select than the actual receptor. Despite the fact that many receptors such as EGFR, ErbB2, and PDGFR have been implicated in tumor progression, targeting any one of these would not preclude

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signaling by other RPTKs, therefore rendering the downstream convergence point a more efficient target.

A second convergent element would be calpain. Obviously other signaling pathways will be deciphered in the future that would also be rationale targets, such as those leading to cytosol translocation, front adhesion, forward protrusion, and so on. Two prime advantages of multiple targets are the ability to use multi drug therapy and avoid acquired resistence to any one particular agent and the option of using sub optimal dosages to avoid side effects. This latter aspect may be key to limiting tumor invasion during a situation in which other motility-requiring phenomena are needed. Two instances would be during postoperative wound repair and during inflammatory responses. In these situations a combination of targets may prove preferential to inhibiting the mode of motility operative during tumor invasion but not significantly impact overlapping modes needed for tissue repair and regeneration. The studies presented in this dissertation contribute to developing therapeutic agents that would better balance the cellular molecules responsible for pathologic effects in a way that would enhance the efficiency of treatment. The near future will undoubtedly witness an increase in the development of such agents for these purposes.

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