In vitro analysis of integrin mediated adhesion, migration, and signaling

William Lathrop Rust
University of Nevada, Las Vegas

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IN VITRO ANALYSIS OF INTEGRIN MEDIATED ADHESION, MIGRATION, AND SIGNALING

by

William L. Rust

Bachelor of Science
Iowa State University
1997

A dissertation submitted in partial fulfillment of the requirements for the degree of

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Examination Committee Chair

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ABSTRACT

In Vitro Analysis of Integrin Mediated Adhesion, Migration, and Signaling

by

William L. Rust

Dr. George Plopper, Examination Committee Chair
Assistant Professor of Biological Sciences
University of Nevada, Las Vegas

Integrins are cellular adhesion receptors whose functions are critical to the progression of solid tissue cancers. The research described here involves four projects aimed at understanding how integrins modulate key elements of carcinogenesis, (adhesion and migration), on laminin basal lamina proteins.

In order to carry out these studies, a novel fluorescence based protocol for quantifying cell migration towards a bound substrate was developed (Chapter 2). This method is more reliable, efficient, and informative than currently available protocols. I demonstrate its use as a screen for migration-blocking chemotherapeutic drugs.

The small heat shock protein 27 (HSP27) is found to be over-expressed in many mammary carcinomas and can be an effective prognosticato of disease recurrence.
following treatment. The mechanisms for this action of HSP27 are not fully understood.

In chapter 3, I demonstrate that over-expressed and activated HSP27 enhances migration towards laminin-5 in breast carcinoma cells through an intracellular signaling pathway involving mitogen activated protein kinase (MAPK/ERK 1/2). In addition, non-activated HSP27 in the same cells confers resistance to a subset of drugs that would halt migration.

In a screen of drugs that block the function of specific intracellular signaling molecules, it was found that an inhibitor of G-protein mediated signaling blocked the integrin induced migration of breast epithelial cells on laminin-5. I demonstrated in chapter 4 that pro-migratory signaling cascades stimulated by the β1 class of integrins are inhibited specifically by interruption of Goi and not Goβ heterotrimeric G-protein isoforms. Furthermore, β1 stimulation caused an increase of an intracellular G-protein effector (cAMP). Artificial manipulation of cAMP mimicked the effects of β1 stimulation on migration of these cells. These data suggested for the first time that G-proteins function in integrin stimulated signal cascades.

Because most cells express several types of integrins simultaneously, in chapter 5 I constructed a model whereby the contribution of individual integrin types to the net effect of integrin engagement could be analyzed. For this I demonstrated that intracellular calcium flux can be used as a reliable downstream marker of integrin stimulated signaling cascades, and that laminin-1 is a good model substrate for stimulating a variety of integrin subtypes. Next I describe the production and testing of six recombinant fragments of laminin-1 for the purpose of producing activating ligands of specific laminin-1 binding integrins.
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ABBREVIATIONS

AC  Adenylate Cyclase
AM  Acetomethyl ester
ADP Adenosine Diphosphate
bp  base pairs
Ca^2+ Calcium ion
CAI Carboxy Amidotriazole
CaMKII Calcium/calmodulin Dependent Kinase II
cAMP cyclic Adenosine Monophosphate
CD  Cell Determinant
Cdk  Cyclin Dependent Kinase
CRAC Calcium Release Activated Channels
DMEM Dulbecco's Modified Eagle Medium
ECM Extracellular Matrix
EDTA Ethylenediaminetetraacetic acid
EHS Engelbreth-Holm-Swarm
ER Endoplasmic Reticulum
ERK Extracellular Regulated Kinase
FAK Focal Adhesion Kinase
FITC Fluorescein-5-isothiocyanate
FN Fibronectin
GTP Guanine Triphosphate
Hsp Heat shock protein
IAP Integrin Associated Protein
Ig Immunoglobulin
ILK Integrin Linked Kinase
IP_3 Inositol-1,4,5-trisphosphate
JNK Jun N-terminal Kinase
kb kilobase
kDa kilodalton
Ln Laminin
mAb monoclonal Antibody
MAPK Mitogen Activated Protein Kinase
MAPKAP Mitogen Activated Protein Kinase Activated Protein
mL milliliter
μL microliter
MMP Matrix Metalloprotease
MTT 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide Thiazoyol blue

ix
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<th>Abbreviation</th>
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<tr>
<td>NAD</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>Nf-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PI-3K</td>
<td>Phosphatidylinositol 3, trisphosphate Kinase</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
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<td>PKG</td>
<td>Protein Kinase G</td>
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<tr>
<td>PLA2</td>
<td>Phospholipase A2</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative Fluorescence Units</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress Activated Protein Kinase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SH</td>
<td>src Homology</td>
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<tr>
<td>TMS</td>
<td>Transmembrane Superfamily</td>
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CHAPTER 1

GENERAL INTRODUCTION

The behavior of all living cells is controlled by three extrinsic factors. These are the conditions of the immediate environment (pH, temperature, salinity, osmolality, irradiation), the static environment (molecules of connective tissue and neighboring cells), and the soluble environment (mitogens, endocrine and paracrine signals, hormones). This also holds true for tumorigenic cells of the human body. Regardless of gene expression patterns or mutations, the progression of human cancers is dependent upon cellular interactions with the microenvironment. Although much research into cancer progression has been accomplished, it remains largely unknown how a tumorigenic cell’s microenvironment enhances or blocks cancer growth. In order to understand the cumulative effect of these extrinsic factors in controlling the progression of human cancers, the impact of each must first be fully understood.

The research described here focuses on understanding the cellular interaction of normal and tumorigenic epithelial cells with a component of the static environment, the basal lamina, in exclusion of all other influences. Specifically, the interaction between the integrin class of adhesion receptors and laminins, the most abundant members of the
basal lamina [1]. Each chapter describes a project aimed at understanding the specific intracellular signaling stimulated by integrin engagement to laminins, or quantifying integrin-mediated adhesion and migration on laminins.

Integrins are not the only receptors by which cells interact with the static environment, but are the major receptors by which the cells of solid tissues interact with the basal lamina [2]. Four other families of adhesion molecules have been identified. These are the cadherins, the immunoglobulin (Ig) superfamily, selectins, and CD44 [3]. Cadherins are a family of transmembrane glycoproteins that mediate cell-cell adhesion via homotypic (cadherin to cadherin) interactions and are fundamental determinants of tissue organization in developing and adult organisms [3-5]. Ig superfamily adhesion molecules are characterized by the presence of immunoglobulin-like domains. This family mediates endothelial cell interactions with lymphocytes, monocytes, neutrophils, and tumor cells that result in adhesion and trans-endothelial migration. Selectins are transmembrane glycoproteins that mediate heterotypic interactions between blood and endothelial cells during lymphocyte homing and leukocyte adhesion. CD44 is a widely distributed integral membrane glycoprotein shown to facilitate lymphocyte homing and T-cell activation, and is the primary cell surface receptor for hyaluronan [3]. Finally, integrins are transmembrane heterodimers that are obligatory on the plasma membrane of every mammalian cell type, and the focus of the majority of cell adhesion research [6].

The importance of integrin function in mammals is evidenced by gene knock-out studies in mice. Deletion of individual integrin subtypes leads to unique phenotypes with little overlap, and most deletions are perinatal lethal. In fact, deletion of a class of integrins (β1) results in embryonic lethality by day five. [7, 8].

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As our understanding of cancer development grows, it has become clear that the integrin family of cell adhesion receptors play an important role in virtually every stage of cancer progression. Although integrins are not oncoproteins, they help modulate the processes of cell growth, death, migration, and invasion, which all impinge on the severity of clinical disease [9]. It is common to find that oncogenic transformation accompanies changes in integrin expression and substrate preference. Several drugs in clinical trials function as integrin antagonists, all having shown promise as anti-angiogenic, anti metastatic, and anti-proliferative compounds in mice models [10-12]. It is from studies of tumorigenic tissues and cells that much of our understanding of integrin function has emerged.

**Basal Lamina**

The basal lamina are sheets of extracellular matrix (ECM) that line all the cavities and free surfaces of the body, forming barriers from one body compartment to another. Made and organized by epithelial cells, the ECM provides an adhesive substrate, transmits signals through adhesion receptors, and binds, stores, and presents growth factors. During embryogenesis, the basal lamina functions to promote proliferation, differentiation, and migration of embryonic cells. In adults, the basal lamina functions as a molecular filter, polarizing epithelial cells, shaping the tissue structures, supporting infiltration of immunocytes, and guiding the migration of regenerating cells following injury [8, 10]. The components of the extracellular matrix are polysaccharide chains of the class called glycosaminoglycans, structural fibrous proteins (collagen and elastin), and adhesive
proteins, (fibronectin and laminin). The polysaccharide chains are usually found covalently linked to protein in the form of proteoglycans.

Laminins are the first ECM proteins expressed in the developing embryo (2-4 cell stage), they are the most abundant basal lamina protein, and they are found in every adult basal lamina [1, 13]. These are a family of heterotrimeric glycoproteins composed of a single $\alpha$ (~400 kDa), $\beta$ (~200 kDa), and $\gamma$ (~200 kDa) chain (figure 1). Diversity within the laminin family is achieved by forming different combinations of $\alpha\beta\gamma$ trimers [13]. Currently, twelve laminin isoforms are known. Each chain is composed of 2-4 N-terminal globular domains connected to a large coiled-coil region. The $\alpha$ chains have an additional C-terminal domain composed of five repeating globular subdomains. The three chains interact solely at the coiled-coil region, and are stabilized by disulfide bonds, forming an overall cross-shaped structure [13].

The laminins of interest for this study are laminin-5 and laminin-1. Laminin-5 is abundantly expressed in the basal lamina of breast tissue, and is the preferred adhesive substrate of many breast carcinoma cells. In addition to being expressed as several splice variants, laminin-5 is modified in vivo by proteases which recognize cleavage sites on the $\alpha$ and $\gamma$ short arms [14]. Modification of this isoform is shown to alter the migratory and invasive properties of tumorigenic epithelial cells and is found at the leading edge of metastatic invasion [13]. Laminin-1 is the first discovered and best studied of all the laminin isoforms. It is isolated from murine Engelbreth-Holm-Swarm (EHS) cells and is commercially available. This isoform is used in our studies as a model substrate for that research advantage.
Figure 1. Structure of laminin-1.
Integrins

*Integrins are the Major Class of ECM receptors*

Integrins mediate adhesion to extracellular matrix proteins and, in a few cases, cell-cell adhesion. There are currently 18 α and 8 β subunits known which interact to form at least 24 αβ heterodimers. Most cells express more than one integrin isomer, which are loosely distributed over the cell’s surface. Integrin expression is different between cell type, and changes with developmental stage and physiological conditions within a cell type [15, 16]. This family of receptors can be classified into three subfamilies. The β1 integrins generally mediate interactions between cells and ECM. The β2 integrins are restricted to leukocytes and are typified by having other cell surface proteins as their ligands. The β3 integrins are almost exclusively expressed on platelets and megakaryocytes and act as important mediators of platelet adhesion [6].

Excepting the fibronectin receptor α5β1, all integrins recognize and bind to more than one ECM molecule. Each ECM molecule is also known to be bound by more than one integrin. Although it is impossible to predict an integrin binding site based on sequence, an acidic residue is common to all known binding sites, and many contain the sequence RGD [12].

Integrins on resting cells do not adhere strongly to their ligands, but are rapidly and reversibly activated by cellular agonists. The affinity of integrins for their ligand is enhanced by conformational changes to their dimeric structure. Adhesive strength is increased through lateral clustering of integrins at points of cellular contact, a process termed avidity [6, 17].
Integrins are heterodimeric glycoproteins

Both α and β subunits are composed of a large, stalk like C-terminal extracellular domain, a single transmembrane segment (~20 aa), and with the exception of β4, a small (~250aa) globular cytoplasmic domain stabilized by intrapeptide disulfide bonds. The α chain globular head is composed of a sub-domain of seven homologous 60aa repeats that are predicted to form a β propeller structure containing three or four EF hand type divalent cation binding motifs. Nine α subunits have an additional domain termed the I domain which is the site of a Mg/Mn metal ion-dependent adhesion site (MIDAS) [12, 17, 18]. In addition, all integrin α subunits contain a highly conserved KXGFFKR motif adjacent to the transmembrane domain that is the proposed binding site for several integrin binding proteins [19, 20]. All β chains have a conserved N-terminal globular domain of ~240aa which is similar to the α I subdomain. The β I-like domain contacts the β-propeller domain of the α chain and is responsible for the association between the α and β chains through stabilizing salt bridges involving the cation binding motifs [12, 17].

Although exceptions are easily found, the extracellular domain of the α chain is thought to confer ligand specificity, while the cytoplasmic domain of the β chain is implicated as the major binding site for interacting cytosolic proteins [12, 19]. Two of the cytosolic proteins that are thought to regulate the activation state of the extracellular domains are the Ras family of GTPases and protein kinase C (PKC) [6, 21].

A common, although unproven, model for integrin activation presumes that ligand recognition involves the close association of the C-termini of the α and β subunits. Like the action of opening scissors, this forces a break in the salt bridges holding the
cytoplasmic domains in contact, opening up binding sites for integrin associated cytoplasmic proteins [15].

At sites of integrin activation and clustering, protein aggregates termed focal complexes and focal adhesions assemble on the intracellular surface. The types of proteins that form these complexes can be grouped as either structural, which form links to the actin cytoskeleton, or signaling, which include a variety of kinases and adapter molecules linking integrins to still more kinases, members of the GTPase families, phosphoinositide signaling, and ion release (Fig. 2) [20, 22, 23]. The structural components are talin, α-actinin, vinculin, and filamin. Talin, α-actinin, and vinculin are capable of binding F-actin, while filamin is an actin filament cross linking protein. Of these, only α-actinin is shown to bind directly to integrins, while the others are capable of binding each other and members of the signaling component [18, 24, 25]. These complexes are sites where the cell can generate tension with respect to its surroundings, allowing the cell to alter its shape and carry out complex processes such as migration and cell division [12, 26].

The signaling proteins found at focal complexes and focal adhesions associate with integrin cytoplasmic domains to form a nexus for stimulating intracellular signaling cascades. At these sites, signaling from outside to inside the cell occurs which contributes to the regulation of diverse cellular processes including entry into the cell cycle and programmed cell death (apoptosis), gene transcription, regulation of intracellular pH, differentiation, and migration. Signaling through integrins from the inside to the outside of the cell regulates adhesive strength through affinity and avidity modulation, and helps
Figure 2. Model of signaling complex (A), and structural complex (B) formed at focal adhesions.
in remodeling of the extracellular matrix in the processes of tissue development and tissue invasion.

**Integrins are signaling receptors**

**Outside-in**

Except that integrins have no intrinsic enzymatic activity, there is little justification for drawing a distinction between integrin and soluble factor receptor signaling [6, 15]. Nearly all intracellular signaling events stimulated by growth factors can be at least transiently stimulated by integrins. In fact, many mitogenic responses, including activation of the mitogen activated protein kinases (MAPKs) require integrin activation for a strong or sustained response [27].

Most attention placed on integrin linked signaling focuses on the 125kDa kinase termed focal adhesion kinase (FAK). Although FAK lacks Src homology (SH2 and SH3) binding domains and the ability to anchor to the membrane, it has numerous tyrosine and serine/threonine phosphorylation sites that act as docking sites for the recruitment and activation of several SH2- and SH3-containing classes of signaling molecules. The FAK regulated signaling complexes form a complicated, and seemingly redundant association of adapter molecules that stimulate overlapping signal cascades (Fig. 2) [20, 28]. Talin and paxillin are implicated in the activation of FAK by stimulating its autophosphorylation on Y397. This opens a binding site for the oncoprotein Src. All three of these molecules are implicated in targeting FAK to the focal adhesion. Src also phosphorylates FAK on a number of residues, including Y925. Grb2 binds this motif, and through the action of the guanine nucleotide exchange factor SOS, connects activated receptor tyrosine kinases to Ras/ERK/MAP kinase cascades, [12, 23, 28, 29].
Phosphatidyl Inositol 3-Kinase (PI-3K) also binds to Y397, and mediates cell proliferation, cell migration, and apoptosis through its downstream effector PKB/Akt [16, 21]. P130CAS is an adapter molecule containing SH2 and SH3 binding motifs that also binds to FAK and is phosphorylated by FAK/Src. P130cas is implicated in activating the ERK cascade as well as Jun N-terminal kinase (JNK). Both p130Cas and FAK, through the ERK pathway, are implicated in the activation of several transcription factors [29]. FAK is also implicated in preventing apoptosis through a pathway involving PKC, phospholipase A2 (PLA2), and p53, and in controlling cell cycle progression [27, 29].

Integrin regulated signaling proteins that can be activated independent of FAK include protein kinase C (PKC), integrin linked kinase (ILK), integrin associated protein (IAP) and the tetraspan (TMS4F) adapter proteins [12, 28]. ILK’s only known substrate is integrins, and may function as a bridge to FAK [12]. Although tetraspans can regulate cell motility, their mechanism of action is not understood [30].

Inside-out

The main consequence of inside out signaling is changes in integrin adhesive strength through affinity and avidity modulation. Exactly how this occurs is unknown, but phosphorylation of integrin subunits may be important in both processes [6]. Intracellular calcium is shown to decrease integrin affinity while Ras family GTPases can convert integrins to a high or low affinity state, depending on the cell type [21]. PKC is thought to cause the increase of both integrin affinity and integrin avidity [6].

Integrins Contribute to Cancer Progression

Solid tissue cancers begin as benign aggregates of poorly differentiated cells which have, through a range of possible mutations, lost the normal mechanisms regulating cell
growth and/or cell death. Although not well studied, integrins are shown to influence proliferation through cooperation with growth factor receptors, by specifically causing the transcription of the cyclins and cyclin dependent kinases (Cdks) required for transition past the G1 checkpoint, and by down-regulation of Cdk inhibitors [6, 12, 27].

Programmed cell death, or apoptosis, is regulated by the action of death effector molecules and resistance mechanisms that counteract the pro-apoptotic signals. The ultimate apoptosis effectors are the caspase family of proteases [12]. The fact that most cells deprived of ECM interactions undergo apoptosis, and that integrin ligation can rescue those cells demonstrates that integrins are involved in stimulating the apoptosis resistance mechanisms [12]. Proteins known to be part of those resistance mechanisms include MAPK, NFkB, Bcl-2, PI-3 kinase, JNK and PKB/Akt. In some cell lines, integrins are shown to activate the Bcl-2 gene, while others report that integrins increase the activity of MAPK, JNK, PI-3 kinase, and PKB/Akt [6, 12, 16, 21]. In addition, integrin dependent interaction with the ECM can suppress the activity of caspase-1 [6]. Lastly, cell death by deprivation of engagement of ECM (anoikis) may be an important control mechanism in cancer since carcinoma cells that lose contact with the matrix would die rather than circulate and colonize distant sites [16, 21].

Benign tumors are encapsulated by an organized lattice of basement membrane components. The progression to malignancy, and the clinical diagnosis of malignant disease, is essentially determined by the capability of tumor cells to dissociate, degrade the lattice, and metastasize to other locations within the body. This process, termed the metastatic cascade, begins with the detachment of single tumor cells and active infiltration by those cells to the surrounding stroma where entry into the vasculature and
lymphatic system is possible. Dissociation of individual cells from a tumor mass is regulated by the E-cadherin family of receptors. These are shown to be suppressors of epithelial tumor metastasis. Recently, activation of integrin $\alpha_3\beta_1$ was shown to down-regulate E-cadherin mediated adhesion, causing loss of cell-cell adhesion, junctional communication, and enhancing invasiveness of malignant tumor cells [4].

For invasion by dissociated cells to occur, the ECM that surrounds the neoplastic tissue must be degraded to allow the escape of invasive cells. Integrins participate in the ECM degradation by stimulating the secretion of ECM-degrading proteases. Integrin expression is shown to cause the secretion of the matrix metalloprotease (MMP) family of proteases in vitro, and enhance invasion in vivo, through a signaling cascade involving Ras [12, 21].

Integrins effect the active migration of cells in the following manner: Membrane protrusions (lamellipodia and filopodia) are formed at the leading edge of migrating cells which attach to the substrate by forming focal complexes. The integrin-stimulated signaling molecules conclusively shown to regulate the formation of lamellipodia and filopodia are Rac and Cdc42, while Rho is shown to inhibit the formation of these structures by forming and maintaining stable actin stress fibers. At the trailing end, focal adhesions are disassembled with the help of calpain and gelsolin, and the integrins are recycled to the leading edge. Movement of the cell body is accomplished by contraction of the actin-myosin cytoskeleton which is also triggered by integrin stimulated signal cascades [5, 21, 26, 31, 32]. The rate of cell migration can be plotted as an approximate bell curve versus the extent of cell adhesion. With low-strength integrin binding, migration is relatively slow because weakly attached cells do not generate enough
traction to move significantly. An optimal rate of migration is achieved with increasing adhesion, and further cell attachment impairs motility by binding the substrate too strongly to allow efficient movement [33].

Once in the vasculature, cells are trapped in the microvessels of target organs. For this reason, the lung, liver, kidney, and lymph nodes are common sites of metastasis for most tumors. Once trapped, a percentage of these cells will cross the endothelial cell barrier and, through an act of ECM remodeling and migration as discussed above, invade the host tissue. These cells can be killed by the immune system or remain dormant for many years. Following stimulation by unknown factors, these cells may exit dormancy, begin to divide, recruit blood vessels, and form a secondary tumor.

*Mechanisms of integrin function are not fully understood*

Although much investigation into the mechanisms of integrin function has been accomplished, it remains unknown how signaling specificity by members of this receptor family is achieved. In other words, it is unknown how a few engaged integrin subtypes can regulate the complex behaviors described. It is my hypothesis that, with some overlap, engagement to ECM can stimulate signaling cascades specific to each integrin. This can cause a dynamic intracellular signaling response through mechanisms of integrin crosstalk. Specifically, I hypothesize that laminin binding integrins are capable of independently and simultaneously stimulating several intracellular signal cascades that cooperate with one another to regulate the strength of adhesion and rate of migration. The research described in each of the following four chapters either supports this hypothesis, or provides a method for studying these phenomena.
The following chapter describes a novel protocol for quantifying integrin mediated cell migration. This protocol provides a simpler, more efficient, and more reliable method than previously available. The protocol is presented as a means for measuring the anti-migratory effect of current and experimental anti-cancer chemotherapies. Because most chemotherapies are cytotoxic, and general cellular cytotoxicity is likely to influence cell migration, the protocol includes a simultaneous quantification of cell death amongst the population of cells that have failed to migrate.

Chapter three investigates the influence of heat shock protein 27 (HSP27) on integrin mediated cell migration. HSP27 is a small chaperonin protein implicated in resistance to apoptosis and disease recurrence following chemotherapy. I show that activation of constitutively over-expressed HSP27 enhances migration of a malignant breast cell line \textit{in vitro} and induces resistance to a subset of drugs that inhibit integrin-stimulated migration on the ECM protein laminin-5.

Chapter four provides evidence of a link between $\beta 1$-containing integrins and heterotrimeric G-proteins in the stimulation of breast cell migration on the ECM protein laminin-5. Previous to this study, heterotrimeric G-proteins had not been implicated in pro-migratory integrin-stimulated signaling cascades. The involvement of this class of signaling molecules is demonstrated through the use of specific inhibitors of heterotrimeric G-proteins, artificial modulation of the G-protein effector molecule cAMP, and integrin stimulating antibodies.

In chapter five I build a model to identify mechanisms of integrin cooperation that have the potential of instilling this class of signaling molecules with the specificity required for modulating the variety of effects attributed to them. I describe the production
of recombinant fragments of laminin-1 for the purpose of creating proteins that specifically stimulate individual laminin-1 binding integrin subtypes. In addition, the usefulness of intracellular calcium flux as an indicator of integrin cooperation is demonstrated.

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CHAPTER 2

SCREENING ASSAY FOR PROMIGRATORY/ANTIMIGRATORY COMPOUNDS

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Abstract

Large scale screening strategies aimed at finding anti-cancer drugs traditionally focus on identifying cytotoxic compounds that attack actively dividing cells. Because progression to malignancy involves acquisition of an aggressively invasive phenotype in addition to hyperproliferation, simple and effective screening strategies for finding compounds that target the invasive aspects of cancer progression may prove valuable for identifying alternative and preventative cancer therapies. Here, we describe a fluorescence based automated assay for identifying anti-migratory compounds, with the ability to discern cytotoxic from non-cytotoxic modes of action. With this assay, we analyzed the effects of two drugs on tumorigenic (MDA-MB-435) and non-tumorigenic...
(MCF-10A) human breast cell lines. We chose to compare carboxyamido-triazole (CAI), an experimental compound shown to inhibit migration of various cell types, with tamoxifen, a common preventative and therapeutic anti-cancer compound. Our assay demonstrated that both these compounds inhibit migration at sub-lethal concentrations. Furthermore, CAI was more effective than tamoxifen at inhibiting chemotactic and haptotactic migration of both cell lines at all concentrations tested.

Introduction

Cancer progresses in two general stages. It begins as a carcinoma of clonal, hyperproliferating cells confined to the tissue of origin, which may exist for years as a benign, primary tumor. The conversion of a benign tumor to malignancy involves the acquisition of an aggressively invasive phenotype, wherein the cancer cells leave the tissue of origin and establish new tumor metastases at distant sites. Metastases are first evident at local lymph nodes, with the severest patient prognosis associated with metastases located at distant sites (1).

There are limitations to common chemotherapy regimens. The most common drugs for treating solid tissue cancers are cytotoxic compounds that interfere with the synthesis or function of nucleic acids. These drugs target actively dividing cells of established tumorigenic colonies (2). While many are effective at treating earlier stage cancers, none are curative for later stage disease, when overt metastases are already evident. Because many tumors are not diagnosed until advanced stages, the effectiveness of these drugs is limited (3). In addition, these anti-proliferative agents produce damage to normal tissues, such as immune suppression, mucositis, and hair loss, as well as side effects such as
nausea and vomiting (2, 4). It is important, therefore, to identify alternative, and potentially less toxic treatments for halting the spread of cancer. These strategies focus on preventing the conversion of tumorigenic cells to the malignant phenotype, as opposed to arresting cell growth. For example, recent chemotherapeutic advancements include the introduction of compounds with non-cytotoxic mechanisms of action, such as anti-angiogenic factors, growth factor antagonists, interferons, and agents that induce cellular differentiation (5-8). These non-cytotoxic compounds will potentially be used to prevent the spread of cancer, as well as to increase the effectiveness of cytotoxic drugs. Since the conversion from hyperproliferative to invasive disease typically takes many years, a large window exists for the use of preventative therapies (1, 3).

The cellular changes required for malignant conversion are complex, offering a large and diverse array of potential targets for the development of anti-metastatic drugs. A common theme among these cellular changes is aberrant regulation of cell migration. Examples of these changes include secretion of proteases, decreased synthesis of protease inhibitors; loss of cell-cell contacts; modifications of cell-substrate interactions; and alteration in the response to and production of chemotactic and haptotactic stimuli during tumor induced angiogenesis and/or metastasis (reviewed in 9, 10, 11). The identification of compounds that halt cell migration without inducing cell death may lead to creation of novel compounds that are less toxic than common anti-proliferative agents (3).

Carboxyamido-triazole (CAI) is an example of such an anti-migratory compound, which is currently in stage 2 clinical trials of androgen-independent prostate cancer (12). CAI is an inhibitor of non-voltage gated calcium channels and blocks cell migration or invasion in breast cancer cell lines (13), prostate cancer cell lines (14), ovarian cancer cell...
lines (3), and head and neck squamous cell carcinomas (15). The anti-proliferative effect of CAI is cytostatic, not cytotoxic, as cells will recover after the removal of CAI (14, 16). This indicates that CAI inhibits signal cascades specific to migration and proliferation. Established modes of action for CAI include inhibition of nucleotide metabolism by depleting phosphoribosyl pyrophosphate (16), inhibition of arachidonic acid and phosphoinositide generation (17), and reduction of matrix metalloproteinase activity (13, 18).

While compounds such as CAI hold promise for effective cancer therapy, there is a lack of screening strategies aimed at identifying these non-cytotoxic, anti migratory compounds. Here we present a fluorescence based, high-throughput method for screening potential anti-migratory compounds that is designed to discern cytotoxic from non-cytotoxic mechanisms of action. Using 96 and 24 well migration filter plates with fluorescence-opaque filters, we show that cell migration can be quantitated easily and reliably with the use of the fluorescent dye calcein-AM. Cytotoxicity of compounds tested is determined by labeling non-migrated cells with the fluorescent DNA intercalator propidium iodide. The experimental cells are doubly labeled so that migration and cytotoxicity are measured in the same assay. The use of a high throughput format, and the novel incorporation of screens for both anti-migration and cytotoxicity on the same test cells, represents major improvements in efficiency and cost over traditional trans-filter migration assays.

With this method, we analyzed the effects of CAI and the well-established anti-breast cancer compound tamoxifen on migration and viability of two human breast cell lines. Both compounds inhibited migration of the breast cancer cell line MDA-MB-435 and the
non-tumorigenic breast cell line MCF-10A at sub-cytotoxic concentrations. Our assay demonstrated that, while MDA-MB-435 cells showed resistance to CAI induced cell death, CAI inhibited haptotactic and chemotactic migration of both cell lines more effectively than tamoxifen.

Material and Methods

Materials

MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) was purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Calcein-AM was purchased from Molecular Probes (Eugene, OR). Tamoxifen citrate was purchased from Calbiochem-Novabiochem (La Jolla, CA). Carboxyamido-triazole (CAI) was a generous gift of Dr. Elise C. Kohn (Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, MD). 96 well fluorescence blocking migration plates were provided by Polyfiltronics (Rockland, MA). 24 well fluorescence blocking migration plates were a generous gift of Dr. Nancy Chung-Welch, Becton Dickinson (Bedford, MA). Information regarding the commercial availability of the 24 and 96 well fluorescence blocking migration plates can be found at the Becton Dickinson website (www.bd.com/labware/newprod/fluoroblok.html). Transwell migration plates were purchased from Costar (Cambridge MA).

Tissue Culture

MCF-10A and MDA-MB-435 cells were maintained as previously described (19). Cells were routinely passaged using trypsin/EDTA (Irvine Scientific).
Transwell Migration Assay

Migration assays were conducted as previously described (20), with the following modifications. Transwell filters (8.0 μm pore size, Costar, Cambridge MA) were coated with purified bovine fibronectin, mouse laminin-1 and collagen IV at 20μg/ml in carbonate buffer (pH 9.3), for one hour at room temperature. 120,000 cells were suspended in DME with 292μg/ml L-Glutamine, 100 units/ml penicillin g, 100 mg/ml streptomycin sulfate (GPS, Irvine Scientific, Santa Ana, CA) and 1 mM sodium pyruvate (Gibco BRL, Grand Island, NY), plated on the uncoated side of the filter and incubated in a humidified incubator containing 5% CO₂. Filters were washed twice in phosphate buffered saline (PBS) and the uncoated side of each filter was wiped with a cotton tipped applicator to remove cells that had not migrated. Cells were then fixed in 3.7% formaldehyde for 15 minutes, and stained with crystal violet as in the adhesion assay. Stained cells of four representative fields of each filter were counted at 400X magnification.

96 and 24 Well Fluorescence Migration Assay

An individual filter insert and well of a 24 or 96 well migration plate is indicated in Fig. 1. Both 24 well and 96 well migration plates are designed as a single insert containing all filter wells in one piece. Each filter insert (A) has a UV opaque membrane (B) with 8.0 μm pores. Inserts fit into a well (C) of a 24 or 96 well reservoir plate. The underside of the filters were left uncoated, or coated with 20 μg/ml bovine plasma fibronectin, type IV collagen, or laminin-1 (Gibco BRL, Grand Island, NY) in 0.1 M acetic acid, and allowed to evaporate. The filter insert was submersed in wells of feeder plate containing DME with 1X GPS and 1 mM sodium pyruvate or DME supplemented
FIG. 1. Individual filter insert and feeder well of a 24 or 96 well migration plate. Test cells are loaded into filter insert (A), and allowed to migrate across a fluorescence opaque filter (B) towards media in feeder well (C). Cells are subsequently labeled with fluorescent indicators of cell migration and cell death. Emitted fluorescence of migrated cells is measured from underneath the filter (D). Emitted fluorescence of non-viable cells is measured from above the filter (E).
with 1X GPS and 10% FCS. 100,000 cells suspended in DME with 1X GPS and 1 mM sodium pyruvate were added to the inside of each filter well (80μl and 100μl for the 96 and 24 well plates, respectively). For drug studies, drugs were added to cell suspension immediately before loading cells into migration plate. 30 minutes before the end of the migration assay, calcein-AM was added to the feeder wells at a final concentration of 5μM. Filters and wells were washed twice in PBS to remove excess dye. Fluorescence of calcein-AM labeled cells was measured from the bottom of the plate (D) in a Tecan SpectraFluor plate reader (Research Triangle Park, NC) with 485 nm excitation and 530 nm emission filters. As an indicator of cell death, propidium iodide fluorescence was measured from the top of the filter insert (E).

**Quantification of Cell Death**

Cells grown in 96 well plates were incubated for four hours in 200 μl per well of the appropriate media supplemented with 1.25mg/ml MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue). To release reduced MTT from viable cells, 150 μl of media was replaced with acidic isopropanol (99.7% isopropanol, 0.3% 12.1N HCl) and wells were incubated overnight at room temperature. Absorbance was measured at 570nm vs. 630nm reference using a Tecan SpectraFluor. Quantification of cell death by fluorescence was performed as previously described (21). Briefly, 1.2 mM propidium iodide was added to media of test wells or filters to reach a final concentration of 30μM and incubated for 30 minutes at 37°C. As a positive control, cells were lysed in 1% Triton X-100 (Sigma, St. Louis, MO) detergent during incubation with propidium iodide. Fluorescence of incorporated dye was measured at 560nm excitation, and 645nm emission wavelengths using a Tecan SpectraFluor.
Statistical Analysis

Anova/T-test was performed on data from indicated figures using a 95% confidence interval. All experiments were conducted at least three times with four to sixteen replicates per condition.

Results

Fluorescence of Labeled Cells Correlates Linearly with Cell Number

In order to quantify migrated cells automatically, cells were labeled with the live cell fluorescent indicator calcein-AM, and a standard curve comparing relative fluorescence units (RFU) vs. cell number was obtained (Fig. 2). Specific numbers of pre-labeled MCF-10A cells were plated in a 96 well tissue culture plate and RFU measurements of these cells correlated linearly with cell number (Fig. 2A). Because the plate reader parameters and dimensions of the plates are slightly different in a migration assay, we confirmed this correlation of cell number with RFU on migratory cells on the bottom of the migration filter. MCF-10A cells were allowed to migrate towards serum through transparent transwell filters. Non-migratory cells were removed from the upper wells, and migratory cells measured by both calcein-AM labeling and visual counting of cells in five representative fields of each filter (Fig. 2B). Similar results were observed with MDA-MB-435 cells (data not shown).

A UV Opaque Membrane Allows for Detection of Migratory vs. Non-Migratory Cells

The use of a fluorescence opaque membrane simplifies migration assays and allows separate detection of fluorescently labeled cells on either side of the membrane. The absorption spectrum of the membrane used in our assay is effective at blocking a UV
FIG. 2. Calcein-AM fluorescence correlates linearly with cell number. (A) The indicated number of prelabeled MCF-10A cells was plated in a 96-well plate and fluorescence intensity was measured. (B) Chemotactic migration of 30,000-120,000 MCF-10A cells was measured in a transwell migration filter by calcein-AM fluorescence (y axis) and subsequently the cells were fixed and stained, and representative fields counted (x axis). Error is represented as the standard deviation of 8 wells.
signal through the membrane if either the emission or excitation wavelength of the fluorescent indicator used falls within the absorption band (data not shown and ref. 22). These filters effectively block the fluorescence (>95%) from the opposite side of the membrane of either of the dyes used in our assays (data not shown and ref. 22). This membrane simplifies endpoint analysis by eliminating the need to remove non-migratory cells before quantifying migratory cells. MCF-10A and MDA-MB-435 cells were allowed to migrate through fluorescence opaque filters of 96 or 24 well filter plates towards serum (chemotaxis) or the indicated matrices (haptotaxis), and migration was quantified by calcein-AM labeling (Fig. 3). The data in figure 3 demonstrate that both cell lines migrated more towards fibronectin than other matrices. For this reason, fibronectin was chosen as the haptotactic stimulus for all drug studies. Note that fluorescence values for each cell line cannot be directly compared to each other as different cell lines exhibit different labeling efficiencies. For example, MCF-10A cells emit a fluorescence that can be as much as 40% brighter than that emitted by similarly labeled MDA-MB-435 cells (data not shown).

*Cytotoxic Properties of Drugs can be discerned from Anti-Migratory properties with the Fluorescent Indicator Propidium Iodide*

Because a cytotoxic compound will halt migration by killing cells, the cytotoxic effects of tested compounds must be evaluated in screens for anti-migratory drugs. To this end, we incorporated a fluorescence based cytotoxicity assay that was performed in the upper well of the migration plate immediately following the migration assay. This protocol was adapted from Nieminen et al. (21). The DNA intercalator propidium iodide is a cell impermeant dye whose fluorescence is increased 10 fold upon binding DNA.
FIG 3. MCF-10A and MDA-MB-435 cells each migrate with different strength towards serum and bound matrices. 120,000 cells were allowed to migrate towards the indicated stimulus in 24 and 96 well filter plates with fluorescence opaque filters. Migrated cells were quantified by calcein-AM fluorescence. Error is represented as standard deviation of 16 wells.
The dye is incorporated into membrane compromised, non-viable cells, and is excluded from viable cells. Propidium iodide was added to the upper well of the migration plate thirty minutes before the end of the migration assay. Propidium iodide fluorescence measurements inversely correlated with results obtained with the 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue (MTT) cell viability assay in experiments where cells were exposed to increasing concentrations of sodium azide (Fig. 4). Similar results were seen when cells were exposed to tamoxifen and sodium arsenite (data not shown). Therefore, propidium iodide uptake can be used as a quick and reliable indicator of cell death.

The Potential Anti-Metastases Drug CAI and the Common Anti-Breast Cancer Drug Tamoxifen Inhibited Breast Cell Migration at Non-Cytotoxic Concentrations

To demonstrate the effectiveness of our assay for measuring anti-migratory and cytotoxic properties of drugs, we compared CAI and tamoxifen on a non-tumorigenic and a highly metastatic breast cancer cell line. Both tamoxifen and CAI inhibited migration in a dose-dependent manner at sub lethal concentrations, and CAI was more effective than tamoxifen at halting migration in all cases (Fig. 5). Haptotactic migration on fibronectin was more sensitive to drug effects than chemotactic migration towards serum of both cell lines (Fig. 5, A-D). MCF-10A cells exposed to 10 µM CAI demonstrated a 45% inhibition of haptotactic migration (Fig. 5C). At 20 µM CAI, haptotaxis of these cells was completely abolished, and chemotaxis was inhibited 33%. In contrast, 10 µM tamoxifen inhibited MCF-10A haptotaxis by only 17%. At 20 µM, tamoxifen abolished haptotactic migration of MCF-10A cells and inhibited chemotaxis by 27% (Fig. 5A). MDA-MB-435 cells exposed to 10 µM CAI demonstrated 32% inhibition of haptotaxis and 7%
FIG. 4. Propidium Iodide fluorescence inversely correlates with MTT absorption. 120,000 MCF-10A cells were exposed to the indicated concentrations of sodium azide (NaN₃) for 1 hour. Cell viability was measured by the MTT method (absorbance, left axis). Cell death was measured by incorporation of propidium iodide (PI)(RFU, right axis). Error is represented as the standard deviation of 16 wells.
FIG. 5. MCF-10A (A,C) and MDA-MB-435 cells (B,D) were allowed to migrate towards serum (chemotaxis) or fibronectin (haptotaxis) in the presence of tamoxifen (A,B) or CAI (C,D). After 18 hours, migrated cells were labeled with calcine-AM, and fluorescence quantitated from the bottom. Non-migrated cells were labeled with propidium iodide and fluorescence quantitated from the top. 100% cell death was estimated by lysing cells in filter well with 1% Triton X-100 (TX 100). Error bars represent standard deviation of 4-16 replicate wells. Asterisks represent a significant difference from migration without drugs (p < 0.05).
FIG. 5 - Continued

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inhibition of chemotaxis. At 20 μM, CAI abolished haptotaxis and inhibited chemotaxis by 42% (Fig. 5D). MDA-MB-435 cells exposed to tamoxifen, however, demonstrated non-significant inhibition of chemotaxis and haptotaxis at 10 μM, and, at 20 μM, only 51% inhibition of haptotaxis and 9% inhibition of chemotaxis (Fig. 5B). In addition, MCF-10A cell viability was more sensitive to CAI than MDA-MB-435 cells, as evidenced by increased propidium iodide fluorescence (Fig. 5C,D). Vehicle alone (DMSO, 95% ethanol) did not significantly affect migration in any case (data not shown).

Discussion

Several screening strategies currently exist for the identification of cytotoxic compounds, such as the National Cancer Institute's in vitro anticancer drug discovery screen (24). While unregulated cell migration is a nefarious aspect of cancer progression and other pathological processes such as arthritic inflammation, no high-throughput methods exist to identify inhibitors of cell migration. This is largely because conventional migration assays require excessive manipulation that reduces throughput and thus precludes them from being used in large-scale screens for anti-migration compounds. In addition to the transfilter, modified Boyden chamber assay presented here, other methods for measuring cell migration include two dimensional assays that measure random, non-directional movement on a flat surface and three dimensional migration/invasion assays, where cells are seeded into, or allowed to invade a gelatinous matrix. We chose to use the transfilter migration assay based on the modified Boyden chamber for ease of use and simple determination of migrated vs. non-migrated cells.
In traditional Boyden chamber filter migration and invasion assays, the migratory or invasive cells are fixed, stained, and counted visually under a microscope. In this procedure, only representative fields are chosen and counted, a process that introduces operator bias. In order to discern migratory from non-migratory cells in these assays, non-migratory cells must be physically removed from the upper surface of the membrane. Aside from adding extra manipulation into the assay, the assessment may only be performed once, and it is difficult to assay viability of the removed cells. Cell quantification by fluorescence detection accounts for the fluorescence contribution of every cell, thus omitting operator variability. Other protocols for simplifying the quantification of migrated cells in a modified Boyden chamber apparatus use markers of cell number that are detected after the migrated cells have been lysed. These markers include radioactivity of cell lysate from $^{51}$Cr-labeled cells (25), enzyme activity (26), and absorption of a cellular dye (27). Martin et al. (28) have introduced a protocol for measuring neutrophil migration that also uses calcien-AM for the rapid and sensitive detection of migratory cells. While their protocol takes advantage of a high-throughput format (Neuro Probe, Gaithersburg, MD), this plate does not have a fluorescence opaque filter, thus requiring that non-migratory cells are scraped from the filter before quantification. Ours is the only protocol designed for the detection of cancer cell migration that incorporates both cell death and migration measurements of the same test cell population in the same assay.

Typical plates designed for transfilter migration assays are capable of handling only 12-24 samples each. Improvements to this design presented in this protocol have made the Boyden chamber easier and faster to use. These improvements include increasing the
numbers of test chambers per plate, decreasing the size of the test chambers, and constructing all of the test chambers as one piece, so that all samples per plate can be handled simultaneously.

The complexity of signaling cascades regulating migration indicates that many different strategies can be exploited to disturb the stimulation of migration. The ability to migrate through the tissues of the body is a complicated process involving tissue remodeling, directed movement, and arrest at sites of action (1). Aside from being an integral aspect of cancer metastasis, cell migration also plays an important role in normal cellular processes such as embryonic development, wound healing, angiogenesis, and the immune response. Each step is tightly regulated in normal tissues, and involves the coordination of signals from extracellular matrix receptors, primarily integrins, and growth factor receptors (10, 29). The controls regulating cell movement, while incompletely understood, are diverse, and vary among cell type. Compounds identified by this assay could affect any one of those processes, and identifying the mechanisms of action of those compounds could help clarify how cell migration is accomplished.

In this report, we demonstrate that tamoxifen, an anti-estrogen that competes with estrogen for binding the estrogen receptor (ER) (30), inhibits migration, and causes cell death of estrogen receptor negative (MDA-MB-435) and estrogen receptor positive (MCF-10A) cells. This effect is not surprising since tamoxifen is shown to have an alternate mechanism for affecting anti-proliferation and cytotoxicity, besides binding the estrogen receptor. In fact, ER negative cancer patients can respond to tamoxifen while ER positive patients can be insensitive (31). ER negative breast cells including MDA-MB-435 cells have also been shown to respond to tamoxifen in vitro (31, 32).
The use of a fluorescence blocking membrane suggests the possibility of measuring cell migration over time, as opposed to strictly endpoint analysis. We attempted to measure the decrease in fluorescence from the top of the filter plate and extrapolate those RFU measurements to cell number by comparison to a standard distribution of cells contained in wells of the same plate. To achieve this, the cells would have to be pre-labeled with a bright, stable dye that did not influence cell migration patterns. We assessed four classes of live cell fluorescent dyes for these properties, calcein-AM, SP-DiOC$_{18}$(3), CellTracker Blue, and carboxylate modified fluorescent polystyrene microspheres (all purchased from Molecular Probes, Eugene, OR). Calcein-AM is a low affinity calcium chelator (23), and inhibits migration of our cell lines, probably by interfering with calcium signaling required for migration (data not shown). These data are not surprising, as release of calcium from intracellular stores and opening of calcium channels in the plasma membrane is a consequence of integrin binding and requisite for migration under certain conditions (11, 33, 34). SP-DiOC$_{18}$(3) is a lipophilic carbocyanine membrane probe that is highly fluorescent when incorporated into cell membranes (23). While adhesion and migration are dependent upon membrane integrity, we found that SP-DiOC$_{18}$(3) did not influence migration and only subtly influenced adhesion in our cell lines (data not shown). While this dye showed promise, it exhibited less intense emission than calcein-AM and thus produced less sensitive results. In addition, while the fluorescence of this dye persisted throughout the migration assay, we found fluctuations in emission that interfered with results and were not readily explained. These fluctuations could be due to turnover of the cell membrane during the course of the assay or inherent limitations of the plate reader. CellTracker Blue is a cell-permeant thiol
reactive probe thought to produce fluorescent-glutathione adducts (23). This dye lacked the emission intensity required for these studies (data not shown). Carboxylate modified fluorescent polystyrene microspheres offer another option for labeling migratory cells in a kinetic assay. These microspheres were spontaneously endocytosed and retained in the cytoplasm in our cell lines and do not interfere with invasion in vivo (35). In our assays, however, carboxylate microsphere labeling demonstrated poor reliability and sensitivity, perhaps by being processed and exocytosed from the cells before completion of the migration assay (data not shown). While these dyes are very effective in applications such as fluorescence microscopy where individual cells are visualized, they were not effective for quantifying large cell populations in kinetic migration assays.

In summary, we introduce a fluorescence based, high throughput assay for screening potential anti-migratory compounds. With the use of two fluorescent indicators, this assay is designed to discern between cytotoxic and non-cytotoxic mechanisms of action of migration inhibiting compounds. Using this assay, we demonstrated that both the experimental anti-cancer compound CAI and the common breast cancer treatment tamoxifen inhibited cell migration at sub-cytotoxic concentrations. While MDA-MB-435 cells showed some resistance to cell death induced by high concentrations of CAI, CAI was more effective than tamoxifen at halting migration of both cells lines.

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HEAT SHOCK PROTEIN 27 PLAYS TWO DISTINCT ROLES IN CONTROLLING
HUMAN BREAST CANCER CELL MIGRATION ON LAMININ-5

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Abstract

It has recently been reported that phosphorylation of the small heat shock protein 27 (hsp27) enhances p38 MAP kinase dependent migration of bovine and human vascular endothelial cells. We have examined the role of hsp27 in controlling the constitutive migration of human breast cancer cells on the extra-cellular matrix molecule laminin-5. In a haptotaxis assay, anisomycin- or heat shock-induced phosphorylation of hsp27 enhances migration of MDA-MB-231 breast cancer cells constitutively over-expressing hsp27. Under these conditions, hsp27 redistributes to the nucleus. Unphosphorylated hsp27, which remains in the cytosol, induces resistance to a subset of drugs that inhibit haptotactic migration of these cells. We conclude that hsp27 plays two distinct roles in
controlling migration of breast cancer cells: phosphorylated hsp27 enhances migration, while unphosphorylated hsp27 can sustain migration in the presence of inhibitory drugs.

Introduction

Heat shock protein 27 (hsp27) is a member of the heat shock family of proteins, which confer resistance to a variety of cellular stresses (reviewed in 1,2). Hsp27 over-expression confers resistance to acute heat shock and some anti-cancer drugs (1,3). Hsp27 function is thought to be controlled by its phosphorylation state and intracellular localization, however, the exact mechanism remains unclear.

Following heat shock, hsp27 is phosphorylated and redistributes to the nucleus (1). This phosphorylation is mediated by mitogen activated protein kinase activated protein kinase 2/3, (MAPKAP K2/3) (1), which in turn is activated by upstream kinases ERK 1/2, JNK/SAPK, and p38 MAPK (2-4). Phosphorylation-deficient mutants do not confer heat shock resistance, suggesting that phosphorylation is critical to its ability to protect cells from heat (3).

In vitro, hsp27 inhibits actin polymerization (2,3). In vivo, hsp27 complexes are sometimes associated with actin filaments in motile cell protrusions such as lamellipodia, filopodia, and membrane ruffles (5), suggesting it may play a role in controlling cell motility or cytokinesis, though its exact role in these activities is unclear (6,7).

Extracellular matrix (ECM) proteins play a critical role in controlling numerous functions in virtually all cells, including migration (8,9). The ECM protein laminin-5 is abundantly expressed in the basal lamina of most epithelial tissues, where it promotes growth, differentiation, and migration of epithelial cells (10-12). In the breast, laminin-5
is the preferred adhesive substrate for normal breast epithelial cells and mediates constitutive migration of breast cancer cells (9). The relationship between hsp27 over-expression and laminin-5 mediated migration in breast cells has thus far been unexplored.

In the present study, we show that hsp27 over-expression alone does not affect constitutive migration of MDA-MB-231 human breast cancer cells towards laminin-5. However, phosphorylation of hsp27 by heat shock or MAPK activation enhances this migration over control cells, concomitant with its re-localization to the nucleus. In addition, unphosphorylated hsp27 confers resistance to drugs that inhibit cell migration, and is localized to the cytosol. From these results, we conclude that hsp27 modulates migration on laminin-5 by two distinct mechanisms, that can be distinguished by hsp27 phosphorylation state: phosphorylated hsp27 enhances migration, while unphosphorylated hsp27 offers protection against migration inhibitors. These two distinct functions may reflect the dual role of hsp27 as an actin polymerization modulator and molecular chaperone, respectively.

Materials and Methods

Cells

MDA-MB-231 cells were maintained as previously described (9). DB46 cell line was made by transfecting MDA-MB-231 cells with an hsp27 constitutive expression vector (pβ27), constructed by cloning the human full length hsp27 cDNA (13) fragment into the pHβAPr-1 neo (14) under the control of the β actin promoter as previously described (15). Control cell line DC4 was made by transfecting cells with the same plasmid lacking the hsp27 cDNA sequence.
Materials

SQ22536, KT5823, H89, Bisindolylmaleimide, Genistein, and Anisomycin were purchased from Calbiochem (San Diego, CA). PD98059 was purchased from New England Biolabs, (Beverly, MA); and pertussis toxin was purchased from List Biological Laboratories, (Campbell, CA). Purified laminin-5 was generously provided by Desmos, Inc. (San Diego, CA).

Immunoblotting

Cells were scraped and lysed in RIPA lysis buffer, and lysates subjected to western blot as previously described (16). In this instance, primary antibodies were anti-hsp-27 monoclonal antibody (1:1000 dilution), Stressgen, (Victoria BC, Canada) or anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) monoclonal antibody (1:2500 dilution), New England BioLabs, (Beverly, MA), and alkaline phosphatase conjugated anti-mouse monoclonal antibody was used as the secondary antibody.

Quantification of Apoptosis

Apoptosis was quantified using an ELISA assay kit (Boehringer Mannheim, Indianapolis, IN). Briefly, DC4 and DB46 cells were treated with indicated concentrations of sodium butyrate for 20 hours, then solubilized according to manufacturers protocol. ELISA wells were coated with anti-histone antibody, then loaded with cytoplasmic extracts, and finally, incubated with anti-DNA secondary antibody conjugated with peroxidase. Absorbance of peroxidase substrate was measured at 405nm using a Microplate autoreader (Dynatech MR5000).
Heat Shock Survival

Survival after exposure to 45°C for various time intervals was determined by colony forming assay as previously described (17). Briefly, cells were heat shocked at 45°C, then allowed to grow for 9-12 days. Colonies (>50 cells) were stained with crystal violet and counted. Percent survival is expressed relative to control, unshocked cells.

Transwell Haptotactic Migration Assay

Cell migration was determined as previously described (9) except that cells were stained with 5 μM calcein AM, Molecular Probes, (Eugene, OR) added directly to the migration wells 30 minutes prior to measuring migration. To quantitate migration, the top side of each filter was wiped with a cotton tipped applicator to remove cells that had not migrated through the filter, and fluorescence of the incorporated dye was measured from the filter with a fluorescence plate reader. Relative fluorescence values for each experimental condition are expressed relative to control, untreated samples.

Phosphorylation Assay

Cells were pre-incubated with 100 μCi/ml[^2]P-orthophosphate, NEN, (Boston, MA), in 90% phosphate-free medium for four hours, then subjected to either heat or drug treatment for 30 minutes. Cells were lysed in RIPA, and 15 μg of cell lysates was immunoprecipitated with anti-hsp27 antibody and protein A/G agarose, Santa Cruz Biotechnology, (Santa Cruz CA). Immunoprecipitates were separated by SDS-PAGE (12% acrylamide), and the dried gel was exposed to film. MAP kinase phosphorylation was determined by immunoblotting with monoclonal antibody specific for phosphorylated MAP kinase.
Miscellaneous

Cell adhesion, Immunoprecipitation, and indirect immunofluorescence assays were performed as previously described (16), using anti-hsp-27 monoclonal antibody and anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) monoclonal antibody (New England BioLabs, Beverly, MA) as primary antibodies where indicated. For affinity captured laminin-5 assays, untreated wells were coated with anti laminin-5 monoclonal antibody TR-1 (20ug.ml) in 100mM carbonate buffer (pH 9.3) for one hour at room temperature. After washing and blocking with blotto, wells were incubated for 1 hour at room temperature with 804G cell conditioned medium, thereby allowing for "capture" of soluble laminin-5. Wells were then washed twice with PBST.

Results

The human breast cell line DB46 stably over-expresses hsp27

To examine the effect of hsp27 over-expression on the behavior of human breast cancer cells, we stably transfected clones of MDA-MB-231 cells with the pβ27 plasmid containing full length human hsp27 cDNA under the control of the β-actin promoter (15). Overexpression of hsp27 in transfected clones was confirmed by western blot (Fig.1a), and function of hsp27 in these cells was determined by resistance to sodium butyrate-induced apoptosis and heat shock (Fig 1b,c). We found that DB46 cells expressed 2-3 fold more hsp27 and exhibited increased (10- to 100-fold greater) survival when compared to control DC4 cells. From these results we conclude that DB46 cells constitutively over-express functional hsp27.
FIG 1. DB46 cells overexpress hsp27. (A) Western blot for hsp27 in resting (37°C) and heat shocked (45°C) DB46 cells transfected with hsp27. As a control, parallel analysis was performed on lysates from DC4 cells transfected with a control plasmid. Note that at both 37°C and 45°C, DB46 cells contain approximately two- to three-fold more hsp27 than control cells. (B) Dose response analysis of sodium butyrate-induced apoptosis. Note that DB46 cells exhibit four-fold less apoptosis as measured by ELISA than control cells in response to 10 mM sodium butyrate. (C) Dose response analysis of % survival of cell colonies following heat shock at 45°C.
Hsp27 over-expression does not alter binding of DB46 cells to laminin-5

Because laminin-5 plays a significant role in controlling breast cell growth, migration, and differentiation (8,9), we compared adhesion of DB46 and DC4 cells to laminin-5 using 30 minute cell adhesion assays. We found no difference between cell lines in adhesion to purified laminin-5 (Fig. 2). Laminin-5 was one of the most preferred adhesive substrates compared to additional ECM proteins (laminin-1, collagen IV, fibronectin).

Phosphorylation of Hsp27 by MAP kinase enhances migration of DB46 cells on laminin-5

We determined the impact of hsp27 over-expression on haptotactic migration towards laminin-5 using in vitro Transwell assays. DB46 cells retained the constitutive migration of parental MDA-MB-231 cells on laminin-5, as did control DC4 cells (Fig. 3). Therefore, hsp27 overexpression alone does not significantly affect migration of DB46 cells. Under these conditions, hsp27 was weakly phosphorylated in both DB46 and DC4 cells. However, stimulation of hsp27 by exposure to 41°C, or via activation of MAP kinase with 10 ng/ml anisomycin, led to a significant (29-53%) enhancement of migration in DB46 cells (Fig.3a). Under these conditions, both ERK 1/2 and hsp27 were phosphorylated, as determined by western blot with anti-phospho-ERK 1/2 antibody (Fig. 3b) and immunoprecipitation of hsp27 from 32PO4 radiolabeled cells (Fig. 3c). Addition of the MEK1 (upstream activator of ERK 1/2) inhibitor PD98059 did not significantly inhibit migration under normal conditions. These results indicate that hsp27 phosphorylation by heat or MAP kinase activation enhances migration. While hsp27
FIG 2. hsp27 overexpression does not alter binding of DB46 cells to laminin-5. DB46, DC4, and parental MDA-MB-231 cells were plated on the indicated ECM proteins for 30 minutes, gently washed to remove unbound cells, then fixed, stained, and quantitated as described in Materials and Methods. Ln-1 = Mouse laminin-1, Ln-5 = laminin-5, TR1 = mouse monoclonal anti-laminin-5 antibody, 804G = conditioned medium from 804G cells containing laminin-5. Laminin-5 was captured by affinity method using TR1 antibody as described in Materials and Methods. Data are presented as statistical mean +/- standard deviation (n=16).
FIG 3. Phosphorylation of hsp27 by MAP Kinase enhances migration of DB46 cells on laminin-5. (A) Haptotactic migration assay on laminin-5. During the entire migration period, cells were exposed to mild heat shock (41°C), 10 nM anisomycin, 50 μM PD98059. Results expressed as the statistical mean of the mean measurement from each filter, +/- standard error of the means (n=4). (B) Western blot analysis of ERK 1/2 phosphorylation with anti phospho- MAP kinase antibody. Cells were exposed to the indicated conditions for 30 minutes, then lysed and processed for western blot analysis. (C) Immunoprecipiation analysis of phosphorylated hsp27.
phosphorylation correlated with enhanced migration in DB46 cells, the degree of migration enhancement varied with the relative phosphorylation of MAP kinase.

**DB46 cells resist a subset of migration inhibiting drugs**

Hsp27 over-expression confers resistance to drugs that induce apoptosis and inhibit growth, possibly by modulating intracellular signaling pathways. To test the hypothesis that hsp27 influences laminin-5-associated migration signaling pathways, we repeated our cell migration assays in the presence of several drugs that inhibited intracellular signaling molecules and reduced migration of parental cells.

We found that DB46 cells were less sensitive to a subset of these drugs than the DC4 control cells. Specifically, these cells were less sensitive to the migration inhibiting effects of pertussis toxin (1μg/ml), (74% vs. 51% of control migration), which inhibits activation of the heterotrimeric G protein subunit Gαi3; and completely insensitive to a sublethal dose of a specific inhibitor of PKG (KT5823, 10μg/ml), (96% vs. 43% of control migration) (Fig. 4). In contrast DB46 cells were equally sensitive to inhibitors of adenylate cyclase (SQ22536, 50μM), protein kinase A (H89, 10μM), protein kinase C (bisindolylmaleimide, 10μM) and protein tyrosine kinases (genistein, 50μM) (Figure 4). Thus, hsp27 protection under these conditions is not broadly applied but specific to certain signaling pathways involving at least PKG and Gαi.

**The hsp27 that confers resistance to migration inhibiting drugs is unphosphorylated**

Because hsp27-enhanced migration of DB46 cells is phosphorylation-dependent, we determined the phosphorylation state of hsp27 under conditions where it conferred resistance to migration inhibiting drugs. Identical concentrations of drugs used to inhibit migration were administered to \(^{32}\)PO₄-labeled cells for 30 minutes, and hsp27
FIG 4. DB46 cells resist a subset of migration-inhibiting drugs. Migration assays were performed as in Figure 3, except that cells were plated in the presence of indicated concentrations of inhibitory drugs. Results expressed as the statistical mean of the mean measurement from each filter, +/- standard deviation (n=4).
phosphorylation was determined by immunoprecipitation and autoradiography. Except for modest phosphorylation by bisindolylmaleimide, no drug treatment induced hsp27 phosphorylation over background levels (Fig. 5). These results demonstrate that hsp27 plays two discrete roles in controlling migration in DB46 cells: unphosphorylated hsp27 protects against the migration-inhibiting effects of pertussis toxin and KT5823, while only phosphorylated hsp27 enhances MAP kinase-dependent migration. Percent migration relative to control (37°C, no drugs) is indicated above band for each treatment.

Enhancement of migration by phosphorylated hsp27 correlates with its relocalization to the nucleus

Phosphorylation of hsp27 can affect its intracellular localization (18). Because we observed that hsp27 phosphorylation distinguished between its migration-enhancing or drug resisting effects, we determined its subcellular localization under both conditions. Indirect immunofluorescence microscopy revealed that, in the absence of heat shock or drug treatments, hsp27 was distributed throughout the cytosol with a slight concentration in the perinuclear region (Fig. 6, panel B). Upon heat stimulation or treatment with anisomycin, hsp27 relocated to the nucleus within 45 minutes (Fig. 6, panels F,H). In contrast, hsp27 did not relocalize in cells treated with pertussis toxin (Fig. 6, panel D).

From the above data, we conclude that hsp27 plays two distinct roles in controlling migration of DB46 cells on laminin-5: when phosphorylated, hsp27 concentrates in the nucleus and enhances constitutive migration, while unphosphorylated hsp27 remains distributed throughout the cytosol and offers resistance to a subset of signaling inhibitors that reduce constitutive migration.
FIG. 5. Correlation of migration to hsp27 phosphorylation state. Percent migration data (above) obtained from figures 3a and 4. Immunoprecipitation analysis of phosphorylated hsp27 performed as described in materials and methods using anti hsp27 Ab.
FIG. 6. Localization of hsp27 and f-actin. Fluorescence micrographs of DB46 cells doubly stained for actin (phalloidin) and hsp27. Magnification = 400X.
Discussion

Our data present three lines of evidence that hsp27 acts in two distinct roles to affect cell migration. First, activation of MAP kinase cascades by anisomycin leads to phosphorylation of hsp27 and enhanced migration. While overexpression of hsp27 can enhance cell migration in a phosphorylation dependent manner (19), the upstream signaling mechanisms responsible for mediating this effect are not well defined. In endothelial cells, p38 and SAPK2, but not ERK 1/2, mediate hsp27-dependent modulation of the actin cytoskeleton and chemotactic migration (4,20,21). However, the MAP kinase ERK 1/2 is implicated in ECM mediated events and may be involved in haptotactic migration on laminin-5 (22). Inhibition of ERK 1/2 by PD98059 did not inhibit haptotactic migration on laminin-5, though it's phosphorylation by anisomycin did correlate with enhanced migration. This result does not rule out the involvement of p38 and SAPK, both of which are activated by anisomycin (23).

Second, unphosphorylated hsp27 offers protection against inhibitors of specific intracellular signaling molecules in our cells. Hsp27 selectively protects against cytotoxic compounds, some of which stimulate intracellular signaling cascades (24, 25), perhaps by acting as a molecular chaperone (24); this protection is not always phosphorylation dependent (26). In our cells, hsp27 confers resistance to the repression of migration by inhibitors of PKG and adenylate cyclase, but not inhibitors of PKA and PKC. These results raise the possibility that hsp27 may function as a molecular chaperone or signaling molecule to maintain activity of a subset of signaling pathways required for constitutive migration of malignant tumor cells.
Third, hsp27 redistributes to the nucleus and sites of actin polymerization when phosphorylated, and remains cytosolic when unphosphorylated. This differential distribution correlates with the two distinct functions we observe in our cells, enhancement of migration, and protection against signaling inhibitors. Others have reported similar changes in distribution of hsp27 in response to serum stimulation (2, 18), although re-localization in response to heat shock may be independent of hsp27 phosphorylation (5).

In summary, the most significant finding of this study is that hsp27 affects migration of breast cancer cells by two distinct mechanisms. Further, these mechanisms can be distinguished on the basis of hsp27 phosphorylation state and intracellular localization. Because hsp27 is thought to act as a molecular chaperone, our data support a model whereby unphosphorylated hsp27 sequesters damaged signaling molecules, thereby conferring resistance to drugs that otherwise inhibit haptotactic migration of breast cancer cells. Because phosphorylated hsp27 functions as an actin capping protein \textit{in vitro} (4), this may explain why it enhances constitutive migration of breast cancer cells: In our model, phosphorylation of hsp27, coupled with a haptotactic migration stimulus (e.g., binding to ECM proteins such as laminin-5), may enhance actin filament assembly within the leading edge of migrating cells.

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CHAPTER 4

ANTIBODY INDUCED ACTIVATION OF \( \beta_1 \) INTEGRIN RECEPTORS
STIMULATES cAMP-DEPENDENT MIGRATION OF
BREAST CELLS ON LAMININ-5

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Abstract

The \( \beta_1 \) integrin-stimulating antibody TS2/16 induces cAMP-dependent migration of MCF-10A breast cells on the extracellular matrix protein laminin-5. TS2/16 stimulates a rise in intracellular cAMP within 20 minutes after plating. Pertussis toxin, which inhibits both antibody-induced migration and cAMP accumulation, targets the Goi3 subunit of heterotrimeric G proteins in these cells, suggesting that Goi3 may link integrin activation and migration via a cAMP signaling pathway.
Introduction

Laminins are a diverse group of heterotrimeric extracellular matrix proteins that constitute a major component of the basement membrane of epithelial tissues. The laminin-5 isoform, consisting of the α3, β3 and γ2 subunits, is abundantly expressed in the basement membrane of breast tissue [1] where it plays a role in mammary branching morphogenesis, and adhesion and migration of breast epithelial cells [2].

Evidence from both in vitro and in vivo studies support a functional role for laminin-5 in cell migration of both normal and malignant breast epithelial cells. Our laboratory has previously shown that in vitro, laminin-5 is the preferred adhesive substrate for breast epithelial cells [1]. In haptotactic migration assays, non-tumorigenic breast cell lines fail to migrate significantly on laminin-5, whereas laminin-5 supports migration of highly malignant breast cell lines. In vivo, laminin-5 expression is enhanced in invading regions of metastatic breast tumors[3]. In addition, an altered conformation of laminin-5, resulting from proteolytic cleavage of the γ2 chain by matrix metalloprotease 2, is found at sites of tissue invasion, and this cleavage stimulates migration of otherwise non-migratory breast cells in vitro [4]. Laminin-5 may contribute to the progression of tumorigenic breast cells from the stationary to malignant phenotype by stimulating enhanced migration of these cells.

Cells interact with laminins primarily through integrin receptors [5]. Ligand induced signal transduction by integrin/laminin binding regulates intracellular pH, tyrosine phosphorylation, inositol lipid metabolism, and calcium (Ca++) oscillations [6]. Signaling molecules known to associate with integrins receptors include protein tyrosine kinases, serine/threonine kinases, phospholipid kinases and lipases, ion channels, and members of
the rho family of small molecular weight GTP binding proteins [6]. Laminin-5 is recognized by the α3β1, α6β1 and α6β4 integrin receptors in a number of cell types, and the functional consequence of these interactions depend on the integrin receptor engaged. For example, ligation of laminin-5 with the α6β4 integrin receptor supports branching morphogenesis and hemidesmosome formation in breast epithelial cells [2], while interaction with α3β1 integrin supports migration of these same cells in vitro [7]. Little information is currently available on the specific signaling pathways triggered during these events.

While investigating the role of the α3β1 integrin in motility of breast epithelial cells, we observed that haptotactic migration of the immortalized breast epithelial cell line MCF-10A on laminin-5 was stimulated by direct activation of the β1 integrin receptor with the β1-activating monoclonal antibody TS2/16. Migration was dependent on intracellular cAMP signaling, and TS2/16-promoted a rise in intracellular cAMP levels that occurred 20 minutes after plating on laminin-5. Migration and cAMP accumulation were inhibited by treatment of the cells with pertussis toxin, a compound that inactivates the α subunit of the inhibitory class of heterotrimeric G proteins via ADP-ribosylation. We show that the Gαi3 isoform is a target for ribosylation by pertussis toxin in these cells. Together these data present evidence that the β1 integrin participates in the regulation of MCF-10A cell migration on laminin-5 through a cAMP-signaling pathway involving Gαi3. This is the first description linking integrin activation to signaling through heterotrimeric G proteins.
Materials and Methods

Cells

MCF-10A cells were maintained in DFCI medium according to Band and Sager [8]. MDA-MB-231 cells were cultured as described [1]. Rat 804G cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum and 1X Glutamine Pen-Strep solution (Irving Scientific). 804G cell conditioned medium was collected after 3 days of culturing and was clarified by centrifugation at 1500 x g.

Reagents

Mouse monoclonal antibodies against human integrin α3 (clone P1B5) and β1 (Clone P4ClO) were purchased from Gibco (Gaithersburg, MD). Mouse monoclonal antibody clone P5D2 against human β1 integrin was purchased from Chemicon (Temecula, CA). Purified rat anti-mouse β1 antibody 9EG7 was purchased from Pharmingen (San Diego, CA), and dialysed against PBS to remove sodium azide. Mouse monoclonal anti-human, activating β1 integrin antibody TS2/16 (in ascites form) was generously provided by Dr. Martin Hemler (Dana Farber Cancer Institute, Boston, MA). Anti-rat laminin-5 monoclonal antibody TRl was produced in this laboratory [9]. Both TS2/16 and TRl were purified with a protein G affinity chromatography kit (Pierce, Rockland IL). SQ22536 was purchased from Biomol (Plymouth Meeting, PA) and pertussis toxin from List Biological Laboratories, Inc. (Campbell, CA). 8-Bromo-cAMP, dibutryl cAMP, H-89, and forskolin were purchased from Calbiochem (San Diego, CA).
Adhesion and migration assays

Adhesion and migration assays were performed as previously described [1]. For anti-integrin antibody blocking experiments, antibodies were incubated with cells for 30 minutes before adding to assay wells, and were present throughout the assays.

cAMP determination

Cells were collected by brief trypsinization, blocked with trypsin inhibitor, washed in DMEM, counted, resuspended at $1 \times 10^6$ cells/ml, and incubated at 37°C in migration medium/1 mM isobutylmethylxanthine (Sigma) to block phosphodiesterase activity. After 30 minutes, anti-integrin antibodies (TS2/16 or P5D2) were added, and cells were incubated at 37°C for an additional hour. Control cells were suspended in DMEM/1 mM isobutylmethylxanthine alone during this time. Cells ($1 \times 10^6$/plate) were then plated on 35 mm dishes coated with affinity-captured laminin-5 [1] and incubated at 37°C for the indicated times. Cells representing the 0 time point were immediately retrieved from the dishes, collected by centrifugation, and lysed in cold cAMP extraction solution (95% ethanol, 5% 0.1 N HCl). After 10, 20, 30, and 90 minutes non-adherent cells were aspirated, plates were washed with PBS, and cAMP extraction buffer was added to the adherent cells. The PBS washes from each plate were centrifuged to collect loosely adherent cells, and these were added back to the appropriate extraction. All samples were kept on ice in cAMP extraction buffer for 2 hours, then centrifuged to pellet precipitated protein. Protein was dissolved in 0.1 N NaOH and concentrations were determined with the BCA microassay (Pierce). Supernatants were evaporated and cAMP measured using a cAMP EIA kit (Perseptive Diagnostics, Inc., Cambridge MA) as directed by the
manufacturer. cAMP amounts were normalized to total protein in each sample and expressed as fmol/µg protein.

**ADP ribosylation assay**

Membranes were isolated from MCF-10A cells by lysis in ice-cold 10 mM HEPES pH 7.5, 3 mM MgCl₂, 2 mM EDTA containing 10 µg/ml leupeptin, 2 µg/ml aprotinin, and 0.5 mg/ml Pefabloc SC (Boehringer Mannheim Biochemicals, Indianapolis, IN). Cells were scraped, centrifuged to pellet nuclei, and the supernatant was collected. Membranes were pelleted from supernatant by centrifugation at 13,000 x g for 30 minutes at 4°C; and the pellets were resuspended in lysis buffer. Protein concentrations were determined by BCA protein assay (Pierce).

**ADP-ribosylation and Immunoprecipitation:**

ADP-ribosylation reactions were performed as described [10]. Final reaction conditions were as follows: 100 µg membrane protein was suspended in 20 mM thymidine, 1 mM ATP, 1 mM GTP, 1 mM EDTA, 20 mM HEPES, pH 7.5 with or without 7.5 µg pertussis toxin (activated prior to experiment by incubation for 10 minutes at 37°C in 20 mM DTT, 20 mM HEPES, pH 7.5) and 25 µCi ³²P-NAD (Specific activity = 30 Ci/mM, New England Nuclear catalog # BLU023). Reactions proceeded for 45 minutes at 30°C and were stopped by chilling to 4°C followed by a wash with 20 mM HEPES pH 7.5, 1 mM EDTA and 1 mM DTT. For SDS-PAGE analysis, membranes were solubilized in 50 µl Laemmli sample buffer (LSB), heated for 5 minutes at 100°C and separated on a 12% SDS-polyacrylamide gel. ³²P-labeled proteins were detected by autoradiography of dried gels using Kodak X-Omat AR film with intensifying screens. For immunoprecipitations, ribosylated membrane proteins were solubilized in RIPA
buffer containing protease inhibitors and were incubated with the following G-protein α
subunit-specific peptide antibodies: I-20, specific for Gαi-1; C-10, specific for Gαi-3
(Santa Cruz Biotechnology). Immune complexes were captured by incubation with A/G
agarose (Santa Cruz Biotechnology), solubilized by boiling in LSB and analyzed by
SDS-PAGE as described.

**Results and Discussion**

*The β1 integrin activating antibody TS2/16 stimulated MCF-10A migration on laminin-5*

The non-tumorigenic breast cell MCF-10A remains statically adherent to laminin-5
via the α3β1 integrin [1]. In haptotactic Transwell filter migration assays, these cells
demonstrated only modest migration towards laminin-5. When pre-incubated with
TS2/16, however, MCF-10A cells increased their migration in a dose-dependent manner
towards laminin-5 (Fig 1). TS2/16-treated cells also exhibited increased adhesion to
laminin-5 (Fig. 2). These effects are not observed with other β1 targeting antibodies
(P5D2, 9EG7, Fig. 1; 9EG7, Fig. 2) or with TS2/16 on other substrates (data not shown).
TS2/16 therefore stimulated a signaling pathway that, concurrent with laminin-5 binding,
led to enhanced cell migration. This pathway is dependent upon binding of the α3β1
integrin, as pretreatment of the cells with the α3 integrin-blocking antibody P1B5
completely blocked TS2/16-stimulated migration on laminin-5 (Fig 1).

In each experiment, maximum stimulation of cell migration was observed when cells
were allowed to migrate towards a gradient of fetal calf serum. This control was included
in each migration assay to indicate the dynamic range of migration response in each
population of cells. It is likely that this chemotactic migration was stimulated by the
FIG. 1. The integrin activating antibody TS2/16 stimulates migration of MCF-10A breast cells on laminin-5. Indicated concentrations of TS2/16 were added to MCF-10A cells in a minimal medium lacking serum or other growth factors (MM) 15 minutes prior to plating in laminin-5 migration assays, and migrated cells were counted 18 hours later. As controls, cells were plated in the presence of 10% serum, irrelevant mouse ascites (FM3 ascites), antibody purified from irrelevant ascites (FM3 pure), or non-fat dried milk (blotto). Results are expressed as the mean of eight measurements on two filters using 300 X magnification, +/- standard deviation.
FIG. 2. TS2/16 antibody increases adhesion of MCF-10A cells to laminin-5. Cells were incubated in MM with 50 μg/ml of TS2/16 or 9EG7 antibodies for 15 minutes, then were plated on affinity-captured laminin-5 for 30 minutes and adhesion quantified by measuring absorbance of crystal violet-dyed cells at 595 nm. Affinity capture was accomplished by successive addition of indicated concentrations of TR1 antibody, blotto, and 804G-conditioned medium containing soluble laminin-5. As a control (CTL), cells were incubated with no antibodies prior to plating. Results expressed as statistical mean +/- standard deviation (n=8).
growth factors present in fetal calf serum, as serum-induced migration was inhibited by greater than 80% when cells were preincubated with antibodies that block the function of the epidermal growth factor receptor or drugs that inhibit tyrosine kinases (G.E. Plopper, unpublished data). It is therefore likely that serum was a stronger pro-migratory stimulant than TS2/16 because it activated several signaling pathways stimulated by soluble growth factors, while TS2/16 targeted integrin-associated signaling pathways.

The strength of cell adhesion to extracellular matrix ligands varies over a wide range, and is under the control of both intracellular and extracellular cues. Work by Lauffenburger [11] suggests that very tight or very loose cell adhesion to matrix proteins will not support cell migration, and that migration occurs only when a medium-strength of adhesion is achieved. Thus, varying the potency of adhesion of integrin receptors for their ligands may be a critical step for regulating cell migration. It is possible that TS2/16 stimulated migration in these cells by changing the strength of adhesion between α3β1 integrin and laminin-5, either directly or via activation of internal signaling pathways.

Alternatively, it is plausible that TS2/16 induced a conformational change in the β1 integrin that mimicked binding to a pro-migratory form of laminin-5, such as those created through proteolytic processing. For example, cleavage of the γ2 subunit of laminin-5 creates a conformation on which MCF-10A cells migrate constitutively [4, 12]. A pro-migratory laminin-5 can be converted to one that inhibits cell migration through cleavage of the α3 chain [13]. In both instances it is assumed that proteolytic processing masks or unmask a pro-migratory domain on the intact laminin-5 trimer. This theory is also supported by studies showing that integrin activation by TS2/16 will rescue the growth of MCF-10A cells inhibited by treatment with laminin-5 blocking antibodies [14].
MCF-10A cell migration on laminin-5 is modulated by cAMP

To define the mechanisms by which TS2/16 stimulated MCF-10A cell migration on laminin-5, we added inhibitors of known signaling molecules to antibody-stimulated cells in haptotaxis migration assays. We found that SQ22536, an inhibitor of adenylate cyclase, and H-89, an inhibitor of cAMP dependent protein kinase, completely blocked TS2/16 stimulated migration on laminin-5 (Fig. 3). In addition, pharmacological enhancement of cAMP levels with either forskolin or the non-hydrolyzable cAMP analogs 8-bromo-cAMP and dibutyryl cAMP were sufficient to enhance migration of MCF-10A cells on laminin-5 to levels stimulated by TS2/16 (Fig. 3). Prolonged (18 hour) exposure to pertussis toxin, a compound that inhibits the cAMP signaling pathway mediated by the Gαi class of signaling proteins, abolished migration on laminin-5 (Figure 4) and reduced cAMP levels in MCF-10A cells over the same time course (Figure 5B). These data established that cAMP was required for enhanced migration of MFC-10A cells on laminin-5.

Because adenylate cyclase activity is governed by different classes of heterotrimeric G proteins we exposed MCF-10A cells to pertussis toxin (an inhibitor of the Gαi class) and cholera toxin (an activator of the Gαs class). While both pertussis and cholera toxin partially blocked serum stimulated migration of MCF-10A cells (approximately 50%), only pertussis toxin blocked TS2/16 stimulated migration on laminin-5 (Fig. 4). These data demonstrated that the specific pathway triggered by TS2/16 and laminin-5 was susceptible to regulation by Gαi rather than Gαs proteins, and again suggest that serum-stimulated migration resulted from activation of multiple signaling pathways, some of which utilize cAMP as a second messenger.
FIG. 3. Enhanced cAMP levels induce migration of MCF-10A cells on laminin-5. Cells were incubated in MM supplemented with 50 μg/ml TS2/16, 50 μg/ml P1B5, 250 mM SQ22536 (SQ), 4 μM H89, 5 nM forskolin (FSK), 500 μM dibutyryl cAMP (db cAMP), or 500 μM 8-bromo-cAMP (8-Br-cAMP) for 15 minutes prior to adding to laminin-5 migration assays. As a control, cells were plated in the presence of serum or in MM on filters lacking laminins (blotto). Results are expressed as in Fig. 1.
FIG. 4. Pertussis toxin inhibits TS2/16-stimulated migration on laminin-5. MCF-10A cells were suspended for 30 minutes in MM supplemented with either 10% serum or 50 μg/ml TS2/16. 100 ng/ml pertussis toxin (PT), 100 ng/ml cholera toxin (CT), were added 15 minutes prior to plating cells in laminin-5 migration assays. As a control, cells suspended in MM were added to filters coated with blotto alone. Results expressed as in Fig. 1.
FIG. 5. Pertussis toxin inhibits a cAMP peak in TS2/16-stimulated cells. MCF-10A cells were suspended in MM supplemented with 20 μg/ml TS2/16 or P5D2 antibodies and plated on laminin-5 for the indicated time, then lysed and assayed for total cAMP content by ELISA assay. As a control, cells were plated in the absence of antibodies (CTL). The experiments were performed (A) in the absence (-PT) or (B) presence (+PT) of 100 ng/ml pertussis toxin. Results are normalized to total cell protein for each time point and represent the mean of triplicate measurements for four experiments, +/- the standard error of the means.
Not all compounds that induced a rise in cAMP stimulated migration on laminin-5. Cholera toxin induced a transient rise in cAMP in MCF-10A cells (G.E. Plopper, unpublished observations), and stimulated growth of these cells when used in small quantities (ng/ml) in the low-serum media for these cells (DFCI medium) [8]. It is generally thought that small quantities of cholera toxin stimulate growth in these cells by activating signaling pathways used by G protein-linked chemokines (Gary Bokoch, Department of Immunology, The Scripps Research Institute, personal communication). Higher concentrations of cAMP (µg/ml) typically used to irreversibly activate Goαs did not stimulate haptotactic migration of MCF-10A cells on laminin-5 (G.E. Plopper unpublished observations), suggesting that while cholera toxin does affect signaling in these cells, the pathways it affects do not play a role in integrin-activated migration on laminin-5.

Chemotactic migration of many cell types is inhibitable by cholera and pertussis toxins [15, 16]. While pertussis toxin allows for unchecked cAMP production in the short term, prolonged pertussis toxin exposure suppressed cAMP levels in our cells, likely because of long-term desensitization of this pathway [17]. Although O'Conner et al. [18] reported that α6β4 expression suppressed cAMP levels in migrating breast cancer cells, no evidence has been published linking cholera and pertussis-sensitive signaling pathways to integrin-activated signaling.

These findings are consistent with our observation that numerous chemokines that modulate cAMP through Goα (bombesin, bradykinin, adrenaline) raised cAMP levels but failed to stimulate migration in our cells (G.E. Plopper, unpublished data). Each of these compounds exerts very distinct responses in breast cells, suggesting that while they share...
cAMP as a second messenger, they must generate specificity elsewhere in their signaling pathways. The specificity necessary to modulate haptotactic migration may be generated by localizing cAMP bursts to specific times and/or locations within a cell, by targeting specific isoforms of adenylate cyclase, or by integrating cAMP bursts with other integrin-associated behaviors (e.g., formation of focal adhesions, generation of cellular tension, activation of signaling pathways linked to migration in other cell types [e.g., those that utilize rho/ras G proteins or focal adhesion kinase]) [19].

Indeed, such integration appears to take place in smooth muscle cells, which exhibit increased migration on collagen upon activation of a cAMP signaling pathway linked to integrin associated protein and α2β1 integrin; this activation also stimulates the mitogen activated protein kinase ERK, and is inhibited by pertussis toxin. [20]. In this study, migration is stimulated upon a reduction in cAMP levels and is inhibited by analogs of cAMP. The differences between these findings and ours may be attributed to differences in cell type, migratory stimulus, migratory substrate, and/or integrin receptor involved: we have observed that inhibition of α2β1 integrin stimulates haptotactic α3β1-mediated migration in our cells, for example (G.E. Plopper, unpublished findings).

TS2/16 stimulated a rise in intracellular cAMP via a pertussis toxin-sensitive signaling pathway

Since pertussis toxin alters intracellular cAMP levels, and cAMP modulation was sufficient to enhance migration in our cells, we examined the levels of cAMP in TS2/16-stimulated cells plated on laminin-5. Within 20 minutes after plating, cAMP levels were raised approximately four-fold in TS2/16 treated cells. This peak occurred within the time frame of integrin signaling [6]. Enhanced cAMP accumulation was specific to
TS2/16, and not a product of integrin clustering, as neither cells treated with the non-
activating β1 antibody P5D2 nor cells plated on laminin-5 without antibodies exhibited
enhanced cAMP production (Figure 5A). Pre-incubation with pertussis toxin completely
eliminated this peak but did not significantly affect basal cAMP levels (Figure 5B).
Concurrent stimulation by laminin-5 adhesion and TS2/16 are required, as cAMP levels
did not change in suspended cells treated with TS2/16 (G.E. Plopper, unpublished). It
appeared, therefore, that the combination of intact laminin-5 and TS2/16 pretreatment
stimulated a signaling pathway involving cAMP that was specifically blocked by
pertussis toxin.

**Pertussis toxin ADP-ribosylated Gaia3 in MCF-10A cells**

Pertussis toxin ADP ribosylates the Gaia class of heterotrimeric G proteins. To
determine the repertoire of Gaia subunits expressed in MCF-10As we performed Western
blot analysis of whole cell lysates and isolated membrane fractions using polyclonal
antibodies raised against specific G protein subunits. These studies revealed that MCF-
10A cells expressed Gaia1 and Gaia3, but not Gaia2 (data not shown). To establish the
targets of pertussis toxin in these cells we carried out ADP-ribosylation assays in the
presence of 32P-NAD. Addition of pertussis toxin specifically induced the ribosylation of
a 43 kDa protein (Fig. 6, lane 2). No 32P labeled proteins are detectable without addition
of pertussis toxin (Fig. 6, lane 1). The molecular weight of the ribosylated protein was
consistent with that of the α subunits of heterotrimeric G proteins. The identity of this
protein was determined by immunoprecipitation of the ribosylated membrane proteins
with Gaia1 and Gaia3 antibodies. Antibody C10, which reacts primarily with Gaia3,
immunoprecipitated a band of 43 kDa (Fig. 6, lane 4). The anti-Gaia1 antibody I-20 failed
FIG. 6. Pertussis toxin specifically ADP ribosylates Goi3 in MCF-10A cells. 100 μg of cell membranes were incubated with 25 μCi $^{32}$P-NAD in the presence (lanes 2-4) or absence (lane 1) of 7.5 μg activated pertussis toxin. Pertussis toxin-treated lysates were immunoprecipitated with anti-Gai1 (I-20, lane 3) or anti-Goi3 (C10, lane 4) antibodies. As a control, Gai1 was immunoprecipitated from $^{32}$P-NAD labeled lysates of pertussis toxin-treated MDA-MB-231 cells (CTL, lane 5). Migration of molecular weight standards is shown at left.
to precipitate any ADP ribosylated proteins in MCF-10A cells (Fig. 6, lane 3), but did precipitate a 43 kDa band from a control cell line, MDA-MB-231 (Fig 6., lane 5).

Therefore, pertussis toxin ribosylated Goi3, but not Goi1 in MCF-10A cells.

In addition to controlling adenylate cyclase activity, Goi3 is associated with and activates amiloride-sensitive Na+ channels [21], which are expressed in many epithelial cells including breast. These channels are also regulated by the actin cytoskeleton [22] and cAMP dependent protein kinase [23], suggesting that Goi3 may link integrin-mediated actin polymerization, cAMP signaling, cAMP dependent protein kinase activity, and amiloride-sensitive channel activation. Curiously, amiloride also suppresses lung metastases from breast tumors [24]; our data suggest that it may do so, at least in part, by inhibiting tumor cell migration.

In conclusion, we report that the β1 integrin-stimulating antibody TS2/16 induced migration of MCF-10A cells on laminin-5 that was dependent upon cAMP linked signaling. TS2/16 also stimulated a rise in intracellular cyclic AMP within 20 minutes after plating on laminin-5. Both the enhanced migration and cAMP peak were inhibited by pertussis toxin. Pertussis toxin targeted the Goi3 subunit of heterotrimeric G proteins in these cells. This evidence suggests that the β1 integrin participates in the control of MCF-10A cell migration on laminin-5 via a cAMP signal pathway regulated by Goi3. This form of signaling, beginning with an external stimulus of the integrin receptor, is referred to as “outside-in signaling” to differentiate it from changes in integrin function resulting from activation of internal signaling pathways [6]. We propose that TS2/16 mimics the effects of proteolytic processing of laminin-5 by forcing the α3β1 integrin into a conformation formed by binding pro-migratory forms of laminin-5. We are

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currently examining the effect of these proteolytic modifications on intracellular signaling activities in MCF-10A cells. Because acquisition of a migratory phenotype is required for malignant progression of tumorigenic breast cells, elucidating pathways involved in enhanced migration of breast may lead to discovery of novel targets for anticancer therapies.

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CHAPTER 5

LAMININ-1 DOMAINS AS SPECIFIC ACTIVATORS OF INTEGRIN SUBTYPES

Abstract

Cells interact with laminin-1 with as many as six different integrin heterodimers. Here I have identified integrins α6β1, α1β1 and potentially α3β1 as laminin-1 receptors used by MCF-10A cells. I hypothesize that MCF-10A cell response to laminin-1 engagement is a product of the cumulative effect of engagement of all three integrins, stimulating at least two separate intracellular signaling cascades. To test this hypothesis, I identified intracellular calcium flux as a downstream marker of integrin signaling response, and demonstrated that clustering of independent integrins and different integrin ligands stimulate unique intracellular calcium fluxes. In order to gauge the impact of each integrin on calcium flux, I constructed six fragments of laminin-1 in a baculovirus system that likely contained integrin binding sites. Each of these were screened for the ability to act as adhesive substrates for MCF-10A cells. My results indicate that recombinant domains of laminin produced by this method are not suitable integrin ligands.
Introduction

It is tempting as a researcher to fit biological molecules into linear cascades of specific function. It is becoming more and more evident, however, that most molecules, especially those involved in signaling, are components of a balancing act between intracellular events whose ultimate outcome can only be predicted by observing the net influence of intracellular conditions within a specific time frame. For example, Ras is recognized as a molecule that promotes cell growth via MAP kinase stimulation. In the absence of PI-3 kinase, or NF-kB activation, Ras stimulation results in the opposite effect, apoptosis [1]. It can therefore be postulated that specific function can not be attached to particular molecules without taking into account other cellular conditions. In order to gain a true understanding of integrin function, the same paradigm must be applied.

To date, a great number of signaling cascades (kinase activation, phospholipid metabolism, small and large G-proteins, ion current, intracellular pH) and cellular functions (proliferation, death, migration, embryogenesis, gene transcription and protein secretion) have been attributed to integrin activation. Since a cell will engage its matrix ligand with only a few integrin subtypes, it is impossible to rationalize the specificity of cellular outcomes based on linear, single-function signal cascades. The hypothesis proposed here is that the consequence of integrin ligation is an emergent property dependent upon the activation state of all expressed integrins. The specificity of integrin function is a result of cooperative balance between integrin stimulated signal cascades.

For this hypothesis to be reasonable, integrins with common substrate specificity must be capable of stimulating different signal cascades, and must be able to
communicate by signal cascade overlap. As an example of similar integrins sending different signals, integrins bound to collagen type IV can either stimulate or inhibit RhoA activity depending on the specific integrins engaged, and time course of engagement [2]. There are several examples in support of the integrin communication by signal cascade overlap. Certain epithelial cells adhere strongly to laminin-5 via α6β1, and weakly to laminin-1, where both α3β1 and α6β1 are engaged. Microinjection of the α3 cytodomain disrupts α6β1 mediated adhesion to laminin-1, indicating that α3β1 transdominantly regulates α6β1 activity [3]. This communication by integrin receptors is also demonstrated in the regulation of phagocytosis and migration by macrophages [4]. In this case, αvβ3 blocks α5β1 function through inhibition of CaMKII. In myocardial muscle cells, transfection with integrin α7 stimulates cell adhesion to laminin-1, but inhibits the ability of native α5 to bind to fibronectin [5].

In order to understand how these emergent properties of integrins are achieved, it is necessary to be able to stimulate individual integrin subtypes alone and in combination, across a variety of cell types. For this I chose to produce recombinant fragments of the likely integrin binding domains of laminin-1, and screened those for the ability to bind one, and only one integrin subtype. Laminin-1 was chosen because it is recognized by six integrin subtypes (α1β1, α2β1, α3β1, α6β1, α7β1, α6β4) and is the most well researched of all the laminin isoforms.

In addition to integrin specific adhesion molecules, it was necessary to identify a marker of dynamic integrin signaling that was likely the result of cooperation between integrin signaling cascades. Intracellular calcium (Ca^{2+}) flux is a likely candidate as it is a consequence of, and required for, integrin mediated adhesion and migration [6 and
Figures 1-3. Increases in Ca\(^{2+}\) are seen upon cell attachment to ECM in platelets, macrophages, neutrophils, osteoclasts, smooth muscle, epithelial, and embryonic stem cells [7, 8, 9]. The complex spatio-temporal aspects of Ca\(^{2+}\) signaling make it well suited for the modulation of highly coordinated processes such as migration, which require an asymmetric regulation of cell adhesion, with formation/strengthening at the front and disassembly/weakening at the rear [10]. Aside from being a factor which regulates integrin affinity and avidity, several proteins which modulate F-actin assembly or maintenance of focal adhesions are also regulated by calcium flux. These include calreticulin, calcineurin, calmodulin, calcium and integrin binding protein, gelsolin, calpain, and calcium/calmodulin dependent kinase II (CamKII) [4, 11, 12, 13]. The variety of mechanisms by which Ca\(^{2+}\) can be regulated also indicate that this is a good molecule for analyzing dynamic regulation by the family of integrin receptors.

The mechanism by which calcium fluxes are propagated and maintained by adherent and/or actively migrating cells is not completely understood. It is generally accepted, however, that intracellular calcium flux results from calcium entry from both internal and external sources. Nearly all cells carry receptor-operated and voltage-operated channels in the plasma and ER membranes, and store operated Ca\(^{2+}\) channels which are activated by a decrease of Ca\(^{2+}\) in the ER [14]. Opening of Ca\(^{2+}\) channels of intracellular Ca\(^{2+}\) stores occurs via inositol-1,4,5-trisphosphate (IP\(_3\)) binding, which is produced by the action of membrane bound phospholipase C (PLC) from the substrate phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)). Sustained Ca\(^{2+}\) release is thought to be achieved by the opening of Ca\(^{2+}\) release activated channels (CRAC) of the plasma membrane [12]. The signaling
molecules PLCy1, CaMKII, calcineurin, and calreticulin are all implicated in stimulating
Ca^{2+} influx through plasma membrane channels [8, 11].

Although it has been reported that integrin-ECM interactions lead to IP_{3} production,
no direct link between integrins and PLC activation has yet been established. A
convincing model of indirect activation, however, has recently emerged. In this model,
phosphatidylinositol-3,4,5-trisphosphate (PIP_{3}) interacts with PLCγ, thus targeting PLCγ
to the membrane and contributing to its activation. PIP_{3} is produced from PIP_{2} by the
action of phosphatidylinositol-3 kinase (PI-3K), an integrin associated kinase. Integrin
binding activates PI-3K through the activation of FAK [12]. Published data in support of
this model include demonstration that PI-3K regulates PLC mediated Ca^{2+} signaling [15],
PI3 kinase targets PLC to the membrane [16], integrin binding activates PI3 kinase, and
PI3 kinase promotes and is required for breast carcinoma invasion [17].

In this research, I identify three integrins expressed by the breast epithelial cells
MCF-10A and MDA-MB-231 as potential receptors for intact laminin-1. I show that
intracellular calcium flux is required for adhesion to laminin-1, and that intracellular
calcium flux is dependent upon the type of matrix and integrin engaged. Furthermore, I
describe the production of six recombinant fragments of laminin-1 by baculovirus
expression system. Unfortunately, none of the recombinant proteins functioned as
efficient integrin ligands, and the potential causes of this result are discussed.
**Materials and Methods**

*Calcium Detection*

Cells were serum starved for 45 minutes and trypsinized until all cells had released the substrate (~10 minutes). Trypsin was neutralized by 5mg/ml trypsin inhibitor (Boehringer Mannheim, Germany) in DME. Cells were then washed in DME and held in suspension for 30 minutes at room temperature in DME supplemented with 50mM HEPES pH 7.4 and 5µM Fluo-3 AM (Molecular Probes, Eugene, OR). 120,000 cells were plated per well in a 96 well plate coated as in adhesion assays with poly-L lysine, matrix proteins, or anti-integrin antibodies. Fluo-3 fluorescence was measured over time at 495nm excitation and 535nm emission in an automated fluorescent plate reader (Tecan SPECTRAFluor, Research Triangle Park, NC) warmed to 37°C. Background auto fluorescence of coated wells containing media only was subtracted from emission fluorescence at each time point.

*Virus construction*

Domains of murine laminin-1 were amplified by polymerase chain reaction (PCR) using sequence specific primers designed to generate single products with endonuclease specific 5' and 3' ends (Table 1). Laminin-1 cDNAs were generously provided by Y. Yamada [18]. The endonuclease digested PCR products were subcloned into the pBACgus-6 transfer plasmid (Novagen, Madison WI) in frame with the gp64 promoter, secretion signal sequence, and either a 5'or 3' 6X Histidine repeat. The cloned product was dideoxy sequenced in both directions to confirm proper insertion of the cDNA into the multiple cloning site of the plasmid. Recombinant baculovirus was generated by cotransfection of the pBACgus-6/domain transfer plasmids with linearized baculovirus
| Domain | cDNA | Vector | Forward Primer | Reverse Primer | 5' RE | 3' RE | Length (bp) | 6X His Repeat | Laminin-1 start |
|--------|------|--------|----------------|----------------|-------|------|-------------|---------------|----------------|---------------|
| aVI    | 1286 (PA2-2) | puc18 | TATGATCCATGGTGGC | GATTAACGAAATTCAAGA | Ncol | EcoRI | 630 | 3' | 112 |
| aVb    | 1285 (PA04) | puc18 | GAGGTTCATGGAGTG | CTTACCCTGAGGTGTC | NcoI | XhoI | 775 | 3' | 1573 |
| G1     | 1601 (A0805) | pcDNA 1 amp | CTAATACGACGCTT | GATAGGAGCTGCTTA | Sadl | Spih | 443 | 5' | 6640 |
| G3     | 1602 (A0805) | pcDNA 1 amp | ACTTGACGTGGGGTG | ACTGCTGGCTAGGCTT | Sadl | April | 749 | 5' | 7378 |
| G4     | 1603 (A0805) | pcDNA 1 amp | GTGATGGCGGGTAC | GTGCTTGCAGAGGC | NcoI | XhoI | 725 | 3' | 8078 |
| G5     | 1604 (A0805) | pcDNA 1 amp | CTTATACCACGCGA | GATTCACGATCCAGA | Sadl | Spih | 579 | 5' | 8764 |

Table 1. Construction of viral transfer plasmid. “CDNA” refers to the plasmid from which the domain sequences were amplified. “RE” = restriction endonuclease. “Length” indicates the size of the PCR product including overhangs which are removed following endonuclease digestion. “Laminin-1 start” refers to the location within the native EHS laminin-1 sequence where the sequence encompassing the produced domain starts.
(BacVector-3000 triple cut virus DNA, Novagen) into *Spodoptera frugiperda 9 (Sf9)* cells maintained in Sf-900 II SFM media (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (FBS, Gemini, CA), 100 U/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate (Life Technologies). Successfully recombinant transfectants were identified by 5-Bromo-4-Chloro-3-indolyl-beta-D-Glucuronic acid (Bio-World, Dublin, OH) digestion and purified by three rounds of plaque purification. Cultured supernatates were analyzed for domain expression by immunoblot analysis with anti-penta His monoclonal antibodies (Qaigen, Valencia, CA). Transfected cell supernatates were subsequently used to generate high-titer stocks of recombinant viruses for future infections.

**Protein production and purification**

Sf9 cells were infected at >10 pfu/cell and incubated at 29°C for 1-2 days in Sf-900 II SFM media supplemented with 100 U/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate. Cells were collected and lysed in insect cell lysis buffer (10mM Tris-Cl pH 7.5, 130 mM NaCl, 1% Triton X-100, 10mM sodium phosphate pH 7.5, 10mM sodium pyrophosphate) supplemented with a 1:40 dilution of protease inhibitor cocktail for use in poly (Histidine) tagged proteins (Sigma-Aldrich, St. Louis, MO) for 45min at 4°C and centrifuged at 10,000G for 45min at 4°C. Ni-NTA agarose beads (Qiagen) were incubated in both cell lysate and cell supernate supplemented with 1mM Phenylmethylsulfonyl fluoride for four hours at 4°C and collected. Beads were pooled and sequentially washed with forty volumes wash buffer (50 mM sodium phosphate, pH 8, 300 mM NaCl) supplemented with 80mM imidazole, and sixty volumes wash buffer supplemented with 5mM imidazole. Protein fractions were collected in elution buffer (50
mM sodium phosphate, pH 8, 300 mM NaCl, 250 mM imidazole) and tested for purity by silver staining (Silver Stain Plus, Bio-Rad Laboratories, Hercules, CA). Each domain used in these studies was >95% pure. Typical yields ranged between 20-100 μg/L.

**Adhesion Assays**

Wells of a 96 well plate were coated for 1 hour at room temperature with laminin-1 or recombinant proteins at the indicated concentrations in elution buffer (see above). Negative control wells were coated for the same time in blotto (phosphate buffered saline (PBS) containing 5% nonfat dry milk in (pH 7.4) and 0.1% Tween 20). Uncoated surfaces were blocked by incubation at room temperature for 1 hour in blotto, and thoroughly washed in PBS. Cells were trypsinized and resuspended in adhesion media (DME, 0.25% heat-inactivated bovine serum albumin (BSA), 50 mM HEPES pH 7.4) at a concentration of 2.4X10^6 cells/ml. Cells were kept in suspension at room temperature for 30 mins with the indicated treatments. One hundred twenty thousand cells were plated per well and incubated in a tissue culture incubator at 37°C for 30 mins. Non-adherent cells were removed by gently rocking the plates upside down in PBS for 15 minutes. Adherent cells were fixed for 15 minutes in 3.7% formaldehyde and stained for 15 minutes in 40% methanol/0.5% crystal violet. Adherent cells were quantified by manual counting using a 20X objective or by measuring absorption at 595nm following cell lysis in 1% SDS. Manual counting was used when it was desirable to preserve the plate for future analysis. Using a 20X objective, cells were counted in a field that appeared to be 50% confluent with cells. Cell number from other representative fields was compared to that number to extrapolate percent confluence.
**Glycosylation Analysis**

To determine the extent of glycosylation, approximately 2μg of each domain protein was cleaved with peptide: N-Glycosidase F (PNGase F) (New England Biolabs, Beverly, MA) according to manufacturers protocol and O-Glycosidase (Roche, Indianapolis, IN). For O-Glycosidase cleavage, proteins were denatured for 10 mins at 100°C in 0.1M β-Mercaptoethanol and 0.1% SDS. After cooling, samples were cleaved with 0.5 mU enzyme in 10% NP-40, 0.1M phosphate buffer pH 6, and 500μg/ml bovine serum albumin. All glycosidase reactions were allowed to proceed for 8 hours at 37°C.

**Immunofluorescence Microscopy**

Coverslips were coated overnight at 4°C with laminin-1 or domain proteins in elution buffer (see above). MDA-MB-231 cells were plated at a density of 6X10⁵ cells/ml in DME supplemented with 50mM HEPES pH 7.4 and 0.25% BSA and allowed to adhere for 90 minutes at 37°C. Adherent cells were fixed with 3% paraformaldehyde and permeabilized in 0.2% Triton X-100 for 15 minutes each. Non specific antibody binding was blocked by 1 hour incubation with 3% BSA in PBS. Focal adhesions were visualized by staining for one hour with 1:400 dilution of anti-vinculin monoclonal antibody (Sigma Aldrich) in blocking solution followed by 1 hour incubation with 1:2000 dilution of rhodamine-conjugated anti-mouse IgG secondary antibody (Molecular Probes, Eugene, OR). F-actin was stained with 1:40 dilution of FITC-conjugated phalloidin (Molecular Probes) following incubation with secondary antibody. Stained cells were visualized with a Zeiss LSM confocal microscope using a 40X objective.
Statistics

Statistical significance was determined by the ANOVA/T-test with a 95% confidence interval using JMP statistical software (Altura Software, Pacific Grove, CA).

Results

Integrin engagement stimulates varied intracellular calcium release

Cells engage ECM matrices with only a subset of expressed integrins, depending on matrix specificity. Although there is some overlap, different subsets of integrins are engaged by each matrix. In order to determine if different matrices stimulate matrix-specific intracellular Ca$^{2+}$ release, identical numbers of MCF-10A cells were pre-incubated with a fluorescent cytosolic Ca$^{2+}$ dye (Fluo-3 AM) and plated on different matrix ligands. Emitted fluorescence was measured over time (fig. 1). Each matrix tested caused the release of a unique concentration of cytosolic calcium, and all were higher than cells plated on poly-L lysine. This is presumably due to the subtypes and quantities of integrins engaged. In order to demonstrate that simple clustering of integrin subtypes is also capable of stimulating variable cytosolic Ca$^{2+}$ flux, cells pre-incubated with Fluo-3 AM were plated in wells coated with antibodies directed against known laminin-1 binding integrins and intact laminin-1. Each of the integrins that influenced adhesion to laminin-1 stimulated an intracellular calcium release that was different from binding of the intact molecule (fig. 2). These results suggest that signaling events downstream of integrin activation synergistically determine the ultimate concentration of cytosolic calcium release upon matrix engagement.
Figure 1. MCF-10A cells plated on the indicated substrates release different concentration of cytosolic calcium over time. Serum starved cells pre-treated for 30 minutes with 5μM Fluo-3 AM were plated at a concentration of 120,000 cells per well in wells of a 96 well plate coated with 20μg/ml of the indicated substrates. Fluorescence was measured every 4 minutes in an automated fluorescence plate reader at 37°C for the indicated time. The results of a representative experiment are shown.
Figure 2. MCF-10A cell plated on anti-integrin antibodies release different concentrations of cytosolic calcium over time. Assay conditions are identical to figure 1. Error bars indicate standard deviation (p = 0.05, n = 4).
Fluo-3 AM is a cell permeant dye whose excitation is increased upon binding free calcium. The acetomethyl (AM) ester is cleaved in the cytosol, thus restricting the dye from entering intracellular calcium stores. While intracellular calcium release occurs with release of distinct concentrations and with frequencies of distinct period [19], this dye is capable of measuring only total release of calcium over time under the conditions used [20]. With a high affinity for calcium, (K_d=325nM), this dye is likely to bind all free cytosolic calcium [20]. Fluorescence measurements therefore reflect accumulation of total calcium released by flux of calcium release over time. The fluorescence measurements reported are not likely to have arisen from changes in pH. The test media was buffered (see materials and methods), Fluo-3 AM is non fluorescent in the absence of calcium and is relatively insensitive to changes in pH. In fact, this dye is used with pH sensitive dyes for simultaneous calcium and pH determinations [20, 21].

At the end of each Ca^{2+} determination assay, cells were washed vigorously in PBS and the number of cells remaining were counted as in adhesion assays (data not shown). The number of adherent cells was roughly inversely proportional to cytosolic calcium release. Laminin-5 is the preferred adhesive substrate of these cells [22], and caused the least release of cytosolic calcium of all the matrices tested (Fig. 1). This suggests that cells loosely adherent or actively migrating within the well released more frequent calcium fluxes, or more calcium per release event.

MCF-10A engagement to laminin-1.

To determine the binding properties of MCF-10A cells on laminin-1, cells were pre-incubated with function blocking antibodies directed against integrins expressed by MCF-10A cells, a chelator of cytosolic calcium (BAPTA-AM), and the PI-3 kinase
inhibitor wortmannin (Fig. 3). Cells were also incubated in heparin sulfate to demonstrate that adhesion to laminin-1 is not dependent upon non-specific charged proteoglycan-heparan interactions. Blocking integrins α1, α6, and β1 inhibit adhesion of MCF-10A cells to laminin-1 indicating that these are the primary integrins that determine the strength of adhesion to laminin-1. α6 is an integrin chain that can form a dimer with either β1 or β4 integrin chains. Because blocking β4 has no effect on adhesion (Fig 3. Lane 14) α6 are binding laminin-1 in the α6β1 configuration. Although not statistically significant, blocking α3 appears to increase the strength of MCF-10A cell adhesion to laminin-1. Integrin α3 is included in these studies as a potential laminin-1 binding molecule due to reports that α3 acts as a negative regulator of α6 activity in other cell lines [3]. MCF-10A engagement to laminin-1 is dependent upon cytosolic calcium as pre-incubation with BAPTA-AM abolishes adhesion (Fig. 3, lane 3). Integrin mediated release of cytosolic Ca^{2+} is partially dependent upon the activity of PI-3 Kinase as wortmannin, a specific inhibitor of PI-3 kinase at the concentrations used, blocks adhesion in a concentration dependent manner (Fig. 3, lanes 4,5).

**Laminin-1 domains as putative integrin engagement sites.**

A comprehensive analysis of structure/function studies conducted with EHS laminin-1 indicates that cellular function can be localized to distinct globular domains on each arm of the laminin-1 subunits (Fig. 4) [23-50]. These studies were conducted with either protease cleavage fragments of laminin-1 or small overlapping peptides derived from the laminin-1 sequence. Only rarely was a specific fragment or peptide implicated as a ligand of a specific integrin subtype. Because the large protease cleavage fragments are likely to be bound by more than one integrin subtype, and because small peptides are unlikely to
Figure 3. Properties of MCF-10A adhesion to EHS laminin-1. 120,000 cells per well were plated with the indicated compounds in wells coated with 20μg/ml laminin-1. Ln-1 = laminin-1 alone, BAPTA = BAPTA-AM, W = wortmannin. Error bars indicate standard deviation (p=0.05, n=8). Diamonds indicate a significant difference from uninfluenced adhesion to intact laminin-1.
Figure 4. Synopsis of structure/function studies conducted with laminin-1 on various cell types from the recent literature.
form conformations similar to the native molecule, I decided to produce several individual domains in a eukaryotic expression system. The baculovirus expression system was chosen for ease of use, protein yield, and because proteins expressed in this system are generally processed in a manner similar to vertebrate cells. Domains αVI (AVI), αIVb (AlVb), G1, G3, G4, and G5 were chosen due to the high probability of having biological activity.

Production of recombinant laminin-1 domains

Six proteins derived from the EHS laminin-1 sequence corresponding to specific globular domains were produced. Based on silver-staining of protein elutions, each protein was purified to greater than 95% purity (data not shown). Protein yields varied from 2-10μg/L. The molecular weight of each protein conforms closely to the predicted size following cleavage of carbohydrate chains (fig. 5). All six proteins are glycosylated via N- and not O-linkages based on gel shifts following cleavage by pan-specific N- and O-glycosidases.

Recombinant domains of laminin-1 produced by baculovirus expression do not support integrin mediated adhesion of MCF-10A and MDA-MB-231 cells.

In order to screen each recombinant protein as an adhesive substrate for epithelial cells, MCF-10A cells were plated in wells coated with purified proteins and intact laminin-1. Due to low concentration of recombinant proteins, wells were coated with equimolar amounts of protein (21.1nM), and not identical masses. Statistically reliable adhesion to recombinant proteins by MCF-10A cells was not demonstrated (Fig. 6). Manganese, which acts to strengthen integrin engagement, did not influence MCF-10A adhesion to the recombinant proteins (data not shown). When pre-incubated with MCF-
Figure 5. Purified recombinant proteins. Proteins were visualized by western blot analysis using anti-His antibody. All proteins are N- and not O-glycosylated as demonstrated by gel shift following cleavage by PNGase F (B), or O-glycosidase (C). A = untreated.
Figure 6. MCF-10A cells do not reliably adhere to recombinant domains of laminin-1. 120,000 cells per well were plated in well coated with equimolar concentrations of intact laminin-1 (20μg/ml) or recombinant domain proteins (0.3-1μg/ml). Error bars indicate standard deviation (p = 0.05, n=18). Diamonds indicate significant difference from adhesion to laminin-1.
10A cells, no recombinant protein influenced adhesion to laminin-1 (data not shown). In order to determine if the failure to bind the recombinant domains was cell line-specific, MDA-MB-231 cells, which bind to laminin-1 more strongly than MCF-10A cells, were also tested for adhesion. As indicated by pre-incubation with function blocking anti-integrin antibodies, MDA-MB-231 cells engaged laminin-1 with the α6β1 integrin (fig. 7, lanes 2-8). These cells did not, however, bind to proteins A6-G4 to any greater extent than negative control (fig. 7 lanes 9-13). These cells did show significant adhesion to protein G5. This binding, however, was integrin-independent as function blocking anti-integrin antibodies failed to influence adhesion (fig 7. Lanes 14-19). Curiously, MDA-MB-231 adhesion to G5 was also independent of proteoglycan-heparan charged interactions (fig 7, lane 20). This indicates that these cells engage protein G5 via a member of another family of adhesion receptors, such as E-cadherin, α-dystroglycan, or CD44.

Because epithelial cell adhesion to substrate normally results in formation of focal complexes and spreading, MDA-MB-231 cells were incubated on laminin-1 and protein G5 for 90 minutes, and visualized by fluorescence microscopy. Focal complex formation was visualized indirectly with anti-vinculin antibody, and actin structure was visualized by staining the actin cytoskeleton with fluorescein-conjugated phalloidin (fig. 8). MDA-MB-231 cells spread and formed distinct focal complexes on laminin-1 (fig. 8, panel A,B). On protein G5, adherent cells remained rounded and did not form any distinct cytoskeletal structures or focal complexes (fig. 8, panels C,D).
Figure 7. Properties of MDA-MB-231 cell adhesion to laminin-1 and protein G5. Assay conditions are identical to figure 3. Error bars indicate standard deviation (p = 0.05, n=4). Diamonds indicate significant difference from adhesion to laminin-1.
Figure 8. Morphology and focal complex formation of MDA-MB-231 cells adherent to laminin-1 and protein G5. Focal adhesions of cells adherent to laminin-1 (panel A) and protein G5 (panel C) were visualized by staining for the focal adhesion marker vinculin. Actin structure of cells adherent to laminin-1 (panel B) and protein G5 (panel D) were visualized by staining with the F-actin binding molecule phalloidin.
Discussion

Failure of the recombinant proteins to support adhesion may be due to altered conformations, low concentration, or artifacts of the baculovirus expression system. Because Sf9 are invertebrate cells, it is possible that the proteins are processed differently than they would be by vertebrate cells. The baculovirus system, however, uses many of the protein modification, processing, and transport systems present in higher eukaryotes, including myristilation, palmitilation, phosphorylation, protein targeting, and cleavage of signal sequences. A difference of concern, however, is that N-linked oligosaccharides in insect cells are only high mannose type. They are never processed to the complex oligosaccharides containing fucose, galactose, and sialic acid seen in vertebrate cells. This is only of concern if integrins require the presence of specific carbohydrate structures for ligand recognition. Non-glycosylated laminins have been reported to be adhesive [51], and non-adhesive (Y. Yamada, personal communication). O-linked glycosylations have been shown to occur in insect cells, but are less well characterized [52, 53].

In addition to altered glycosylation, the low yield of my system could also be responsible for the lack of biological activity of these proteins. In the construction of all recombinant viruses I chose the late gp64 promoter, as opposed to the more common very late polyhedrin (polH) promoter. The gp64 promoter typically produces 60% the yield of the stronger polH promoter but is active at a stage of viral infection when the protein processing machinery required for efficient production of secreted proteins remains intact [54]. The small size of the proteins (16-45kD) also contributed to low yield when measured by weight, as smaller sequences are transcribed no more frequently...
than longer sequences preceded by the same promoter region. Small proteins stored at low concentrations also tend to be less stable over time and less able to withstand freeze-thaw cycles. The low yield necessitated the use of low concentrations for all assays. All intact laminin-1 was used at a concentration of 20μg/ml, while each domain protein was used at ~1μg/ml, which represents a 1:1 domain/intact laminin-1 equimolar ratio (21.1nM). MCF-10A and MDA-MB-231 cells, however, both adhered to laminin-1 plated at 21.1nM to nearly the same extent as when plated in wells coated with 20μg/ml laminin-1 (data not shown).

Although yield with the baculovirus system is unpredictable and varies greatly among different proteins expressed, the yield in our system could be improved by double infection of sf9 cells with viruses containing the domain sequence downstream of both the polH and gp64 promoters. This strategy would extend the time of protein expression during viral infection and take advantage of the stronger polH promoter, but would require constructing, purifying, and amplifying an additional six viruses. A prudent continuation of this work would be to produce proteins from the same DNA sequences described here in another system that may provide a greater yield. Other protein expression systems that may prove successful include direct transfection of bacterial, *Drosophila melanogaster*, yeast, or mammalian cells.

The adhesion of MDA-MB-231 cells to protein G5 may be due to specific laminin-dystroglycan interaction. Dystroglycan is a heterodimeric integral membrane glycoprotein receptor for several ECM molecules including laminins. It is expressed in many epithelial cell types and binds laminin through G domains. In mice knockout studies, it is shown that laminin-1, β1 integrins, and dystroglycan are required at the same
development stage, when the basement membrane first appears [55]. In addition, it is
demonstrated that dystroglycans have affinity for laminin in the low nanomolar range
[56].

The use of synthetic peptides is omitted here as a realistic model of integrin ligands.
Although they are widely demonstrated to effectively inhibit tumor cell adhesion to ECM
and other cells, stimulate anoikis, and decrease tumor cell metastases in vivo, they are not
demonstrated to be activators of integrin function. For example, the binding of a peptide
that blocks adhesion to laminin is itself blocked by only the α6 and not the β1 anti-
integrin antibodies [23]. In addition, most integrins require either magnesium or calcium
in sites away from the binding domain. For this reason, integrin mediated adhesion is
typically blocked by EDTA. Adhesion to integrin binding synthetic peptides are not [23].
This data indicates that the peptides are functioning to block the integrin binding sites but
are not mimicking the binding to an intact native ligand. Lastly, the minimum size of
fibronectin derived peptide that still preserved the total binding activity is a fragment
with a molecular weight of 75kDa [57].

While the recombinant proteins generated proved not to be reliable integrin
substrates, I believe that the generation of recombinant proteins that can activate specific
integrin subtypes by natural receptor/ligand interaction on a variety of cell types will be
valuable integrin research tools. I favor this strategy over the production of transfected
cells that express only defined integrin subtypes as recombinant cells expressing foreign
integrins may not have the cellular machinery required to propagate native integrin-
stimulated signals. In addition, recombinant domains would be more flexible research
tools as they could be used with several cell types as well as in different combinations,
without having to produce doubly and triply transfected cell lines. Lastly, recombinant
domains could be useful for more than just the study of adhesion, but other functions
relevant to cancer progression including cell cycle control, prevention of apoptosis and
migration-associated tissue remodeling [58].

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CHAPTER 6

GENERAL DISCUSSION

My ultimate goal in studying integrin-mediated adhesion, migration, and signaling is to identify targets for the design of chemotherapies that function to inhibit the progress of human cancers. Despite enormous efforts by clinicians and researchers, cancer is the second leading cause of death in the United States [1]. The five year relative survival rate for all cancers combined is 59%. The National Institutes of Health estimate overall annual costs for cancer at $107 billion; $37 billion for direct medical costs (total of all health expenditures), $11 billion for indirect morbidity costs (cost of lost productivity due to illness), and $59 billion for indirect mortality costs (cost of lost productivity due to premature death). Treatment of breast, lung, and prostate cancers account for over half of the direct medical costs [1].

One of the primary reasons for the high morbidity of cancer is the frequent ineffectiveness of currently available chemotherapeutic agents. Most cancer patients are diagnosed at a time when metastases are already evident. At this stage, surgery is never effective at curing disease, and the patient requires adjunct chemo- and/or radiotherapy [2].
Although pathologically and clinically similar, tumors result from distinctive genetic anomalies. For that reason, tumors with identical histologies can respond differently to the same chemotherapies [3, 4]. In fact, no single regimen has ever been demonstrated to be effective in 100% of patients with a given tumor type. Most oncologists, however, choose chemotherapy regimens based on standard tumor classifications obtained from histology and empirical data from clinical trials. For this reason, patients frequently receive treatments of no therapeutic value. These therapies are then altered until an acceptable patient response is achieved. This process can be detrimental by prolonging growth of disease, negatively influencing general patient health and morale, and substantially increasing cost and length of medical care. In addition, many patients who have a high probability of being completely refractory to chemotherapy treatment suffer the side effects of hopeless drug therapies [5].

Aside from early detection, there are two strategies for improving the morbidity of cancer. One is to discover new classes of anti-cancer compounds that attack tumors in unconventional ways. A second is to improve the efficacy of current therapies by tailoring chemotherapy treatment for patients on a case-by-case basis. In order to accomplish these tasks, assay systems need to be developed that can measure the response of human tumors to chemotherapies, develop new techniques for relating preclinical information to clinical results for prediction of future useful clinical agents, and gain a greater understanding of the specific molecular mechanisms that govern cancer progression.
Assay Development

At present, nearly all anticancer chemotherapies function to kill a patient’s tumor cells. But the most dangerous cancer cells do more than grow: they metastasize by migrating away from the initial tumor and spreading throughout the body. Because it quantifies migration as well as cell death, the protocol described in chapter two has the potential to be used as a high-throughput method for discovering novel anti-cancer compounds that function to inhibit cell migration.

In addition, this assay has the potential to be used to tailor drug therapies for individual patients. Distinguishing which population of patients will respond to a specific drug before chemotherapy begins, can help the treating oncologist choose the best therapy route. For example, only 20% of colon cancer patients respond to 5-fluorouracil [6]. Patients whose biopsies are identified as sensitive to 5-fluorouracil have a much better than 20% response and those patients whose biopsies are identified as resistant are, depending on the assay, nearly always unresponsive. If the type of tumor being treated has a low likelihood of response to chemotherapy, such as melanomas, NSCLS and GI neoplasms, a biopsy test can help the oncologist decide whether to treat with chemotherapy at all. If the type of tumor typically does respond to chemotherapy, such as breast, ovarian, and most small cell lung cancers, such a test will help select the most effective, least toxic regimen. If the type of tumor is a generally "curable" cancer such as hematologic neoplasms, the test will help determine the most potentially curative from amongst comparable choices. Also, knowing when to stop treatment in hopeless situations is as important as any other management issue [7, 8].

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The technique of diagnosing a patient’s response to chemotherapy, before chemotherapy begins, is termed an In-Vitro Chemosensitivity Assay (IVCA) [9, 10, 11]. Unfortunately, the widespread use of IVCA has been hindered by failures of early assays to predict patient outcome [10]. This could be due to the fact that all of the currently available IVCAAs measure only the effectiveness of drugs on blocking the growth of malignant cells through quantification of cell proliferation, cell death, or production of intracellular metabolites such as ATP. Therefore, the advantages of the assay described in chapter two also lend it to be used as an IVCA system that is potentially more pertinent, sensitive, and effective than existing diagnostic methods. Convergence of cell migration and cell death data will minimize the occurrence of false positive and false negative responses, and improve the validity of true positive and negative responses. In addition, this assay does not require cell cloning, a process that introduces bias by selecting clonogenic cells from a heterogeneous biopsy.

The main argument against IVCA is that it has never been shown in prospective randomized trials that there is a clear advantage to chemotherapy selected with the benefit of knowledge of the IVCA results compared to chemotherapy selected without knowledge of the IVCA results [7, 9]. This is not because studies were carried out and failed to show a difference. It is because the studies have never been supported and carried out by the NCI, NCI-CCCs and university cancer centers [10]. Arguments in favor include that scores of studies have consistently shown a significantly greater benefit for treatment with drugs that are active in the tests compared to treatment with drugs that are inactive in the tests. Additionally, a growing number of studies have shown a superior survival for patients treated with drugs that are active in these tests [10].
Despite these successes, the full potential of IVCA has not yet been realized. Problems associated with all IVCA techniques can essentially be summed up by stating that no in vitro assay can completely duplicate in vivo conditions. For example, most assays require that the biopsy cells are expanded in culture before testing. This allows selective expansion of subsets of tumor cells so these assays may not be predictive of responses of original tumor cells. Assays using differential cell staining techniques are subject to individual interpretation, and frequently cannot distinguish live cells from cellular debris. Measurements of cellular metabolism cannot discriminate cells that are injured and will recover from those which are truly dead. Assays using cell proliferation as the only valid endpoint have failed to predict clinical outcomes for cancer patients [12]. Lastly, all assays that measure a single biochemical endpoint such as inhibition of DNA synthesis may not accurately reflect cell survival, or may be influenced by cell injury during tissue isolation. Nevertheless, assays such as the one described here need to be continually developed and refined to fully realize a potentially valuable treatment tool.

Basic Research

A major problem facing researchers in the integrin field is that a large number of intracellular molecules interact with integrins, yet no clear identification of their individual functions is known. [13]. For example, focal adhesion formation is not always required for integrin linked signaling, and when it is, proteins involved in numerous signaling cascades are found at focal contacts [14].

In addition, it is difficult to differentiate the function of integrins. Integrin modulated enhancement in metastasis formation may not be due directly to mechanisms of invasion, but due indirectly through control of the cell cycle and prevention of apoptosis. To
further complicate matters, integrin function can be cell-type specific. For example, different tumor cell lines can become more or less tumorigenic with up or down regulation of the same integrin [15]. The research presented here raises similar conundrums.

In chapter three, I identify hsp27 as a factor which resists the effects of anti-migratory compounds on integrin-mediated migration. Although it cannot be ruled out as a promising chemotherapeutic target, this does not imply that hsp27 is a mandatory constituent of pro-migratory integrin-linked signal cascades. In addition to being an anti-apoptotic agent, hsp27 can increase cellular resistance to heat shock and oxidative stress. This protection is the result of several hsp27 actions. It is shown to be a molecular chaperone, to interfere with caspase activation, and influence cytochrome C stimulated signaling [16, S.W. Carper, personal communication]. Its action in migration may be an indirect effect of general survival functions.

In chapter four, I demonstrate that integrins can employ heterotrimeric G-proteins in pro-migratory signaling. It is also known that G-proteins can mediate rapid activation of the MAP kinases ERK 1/ERK 2 [17, 18]. These kinases are integral to pro-migratory signaling and are linked to integrin activation by at least two other separate pathways. Heterotrimeric G-proteins can also influence the activity of FAK associated proteins such as Src, and Pyk2 [19]. Does this imply that, like hsp27, G-protein activation is an indirect consequence of activation of diverse signal transduction mechanisms also involved in pro-migratory signaling? It is also entirely plausible that integrins activate G-proteins by directly stimulating G-protein coupled transmembrane receptors. It seems the only
consistent truth is that cellular behavior is a consequence of cooperation between multiple signal molecules of diverse function.

In order to understand the interrelationships and interdependencies between signal molecules, the researcher must attempt to view a larger role for each molecule besides a spot within a linear activation cascade. This trend is seen in the growth of the genomics and proteomics fields, where a global cellular perspective replaces a single molecule perspective. Once the activators and targets of a kinase are discovered, it follows to be discerned where and when specificity of this signaling occurs. For second messenger flux, such as intracellular ions, cAMP, or phospholipid bursts, the specificity arises from localization within the cell, concentration, and period [20-22]. For example, the catalytic activity of CaMKII is higher upon exposure to calcium flux of a faster period than a slower one [23]. For catalytic enzymes, the specificity likely arises from complex cooperation between target molecules that impact a similar cellular function.

Therefore, the questions that must be answered are: How does cooperation between cascades govern cellular function? What are the critical junctions of this cooperation? Would an upstream or downstream effector be the most successful target for chemotherapy design or genetic manipulation? The hypothesis and model proposed in chapter five attempt to provide an angle for answering these questions in the context of integrin cooperation. While the recombinant proteins proved ineffective, this work demonstrates that integrin cooperation does regulate a dynamic intracellular calcium response. This dynamic response is almost certainly the result of intervening kinases and other second messengers. It follows that more models need to be developed in the future that will give us insight into the cooperative controls of cell behavior. Barring the
discovery of scores more intracellular kinases, this will, in the author's opinion, provide the best explanations for the emergent properties of cell behavior.

The hypothesis presented in the general introduction of this text states, "...laminin binding integrins are capable of independently and simultaneously stimulating several intracellular signal cascades that cooperate with one another to regulate the strength of adhesion and rate of migration." The data presented shows the involvement of two more proteins (HSP27 and heterotrimeric G proteins) in integrin-mediated adhesion and migration not previously reported. A cooperation between integrin-stimulated signal cascades is demonstrated in the regulation of intracellular calcium release reported in chapter four. Furthermore, the partial inhibition of migration by specific inhibitors of intracellular signal molecules (chapters 3,4), or adhesion by specific integrin blocking antibodies (chapter 4), also strongly supports the dependence on communication between integrin stimulated signals in the regulation of these activities. Future exploration of this phenomena should include the production of biologically active laminin domains described in chapter five, followed by analysis of G-protein, HSP27, and intracellular kinase activity in cells plated on those domains. The analysis made here predicts that these proteins will be activated by specific integrins. Lastly, the activation state of all of those proteins at specific points in time will simultaneously control cellular adhesion and migration.

References


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APPENDIX

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