SIGNALING IN CONTEXT: PARSING THE ADHESION-DEPENDENCE OF GROWTH FACTOR SIGNALING

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ABSTRACT

Signaling in context: parsing the adhesion-dependence of growth factor signaling

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Intracellular signaling induced by adhesion and soluble growth factors is a significant contributor to cellular function. The serine/threonine kinase, extracellular signal-regulated kinase (Erk), is a prominent point of signaling crosstalk between adhesion and growth factors. Despite extensive effort, the effect of individual growth factors on adhesion-dependent Erk signaling remains unclear due to considerable protocol differences and qualitative analyses. To address these issues, we developed an experimental technique to compare systematically the crosstalk between adhesion and individual growth factors and a quantitative protocol for measuring the magnitude and dynamics of Erk signaling.

Using these methods, we demonstrate that: (1) Adhesion to fibronectin desensitizes Erk activation for cells stimulated by either PDGF (platelet-derived growth factor) or bFGF (basic fibroblast growth factor), but not by EGF (epidermal growth factor); (2) EGF, but not PDGF or bFGF, induces adhesion-dependent Erk activation enhancement; and (3) for adherent cells, either EGF or PDGF stimulation generates transient Erk activation, while bFGF stimulation mediates sustained Erk activation. This data reveal that there are significant differences in the adhesion-dependence of growth factor signaling. The most striking observation was that adhesion desensitizes cells to low doses of specific growth factors (PDGF and bFGF). Studies conducted to uncover the underlying mechanism(s) revealed that adhesion-mediated desensitization of Erk activation exhibits rapid kinetics and occurs at or above the level of Ras activation, but does not involve Sos hyperphosphorylation.

To further probe the mechanisms responsible for generating different Erk signaling dynamics, we constructed a simple coarse-grain model of Erk activation and deactivation pathways. These pathways are represented by four distinct motifs: activation, constitutive direct-deactivation, feedback-mediated direct-deactivation, and feedback-decoupling. Our model predicts that transient and sustained Erk signaling dynamics require specific combinations of the four signaling motifs. These predictions suggest that differences in the Erk activation motif are most likely responsible for the experimentally observed characteristics of adhesion-dependent EGF-mediated Erk signaling. Furthermore, the model indicates either feedback-decoupling deactivation or direct-deactivation as the mechanisms responsible for the observed transient-versus-sustained signaling dynamics induced by different growth factors.

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ABBREVIATIONS

bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CHO	Chinese hamster ovary
dPBS	Dulbecco's phosphate buffered saline
ECM	extracellular matrix
EGF	epidermal growth factor
Erk	extracellular signal-regulated kinase
Erk_T	total cellular Erk
FN	fibronectin
GH	growth hormone
Grb2	growth factor receptor bound protein 2
GRK2	G protein-coupled receptor kinase 2
HaCat	spontaneously immortalized non-transformed human keratinocyte
HePTP	haemopoietic protein tyrosine phosphatase
HSPG	heparin sulfate proteoglycans
HUVEC	endothelial human umbilical vein
I	active input stimulus
I^-	inactive input stimulus
k_{Act}	activation
k_{const}	constitutive direct-deactivation
$k_{decoupl}$	feedback-decoupling deactivation
k_{dir}	feedback-mediated direct-deactivation
MAPK	mitogen activated protein kinase
MDCK	normal Madin-Darby canine kidney
MEF	mouse embryo fibroblasts
MEKK1	MAP/Erk kinase kinase 1
Mkp1	map kinase phosphatase 1
Mkp2	map kinase phosphatase 2
Mkp3	map kinase phosphatase 3
Mkp4	map kinase phosphatase 4
Pak	P21-activated kinase
pAkt	phosphorylated Akt
PC	phosphatidylcholine
PDGF	platelet-derived growth factor
PH	poly-HEMA
PI-3K	phosphoinositide 3 kinase
PKA	protien kinase A

PKC	protein kinase C
PL	poly-L-lysine
p-Mek^{298}	phosphorylated Mek on Serine-298
ppErk	dually phosphorylated Erk kinase
Rb	tumor suppressor retinoblastoma protein
RGD	Arg-Gly-Asp
RT	room temperature
S	inactive signal
S*	activate signal
SH2	Src homology 2
SH3	Src homology 3
Sos	son-of-sevenless
TCPTP	T-cell protein tyrosine phosphatase
VHR	*vaccinia* H1-related
κ_{Act}	dimensionless activaiton
κ_{const}	dimensionless constitutive direct-deactivation
$\kappa_{decoupl}$	dimensionless feedback-decoupling deactivation
κ_{dir}	dimensionless feedback-mediated direct-deactivation
τ	dimensionless time
θ_I	dimensionless Input

Chapter I. Introduction

Cell adhesion to the extracellular matrix (ECM) is critical for multiple cellular functions including migration, survival, apoptosis, and proliferation. In fact, carcinogenic cells gain anchorage-independence, retaining the ability to proliferate without adhesion to the ECM (Assoian, 1997; Schwartz and Assoian, 2001). Additionally, cell adhesion is important in the area of biomaterial development. Understanding how adhesion affects cellular function is critical in determining the appropriate epitopes required on a biomaterial surface to induce adhesion. Importantly, quantification of growth factor-induced adhesion-dependent signals is a key challenge in assessing the quality and effectiveness of both biomaterials and cancer therapies.

To begin to understand cellular adhesion, it is necessary to discuss the proteins responsible for cell attachment to the ECM. Cell adhesion is mediated by heterodimeric transmembrane proteins called integrins (Asthagiri et al., 1999; Giancotti and Ruoslahti, 1999; van der Flier and Sonnenberg, 2001; Vinogradova et al., 2002; Vuori, 1998). Each integrin contains an alpha and beta extracellular domain that combine to form 24 heterodimers. It is these heterodimers that determine to which ECM proteins the integrin will bind (van der Flier and Sonnenberg, 2001). The ECM protein fibronectin (FN) has been well characterized in mediating cellular adhesion and spreading (Pierschbacher and Ruoslahti, 1984). In fact, the Arg-Gly-Asp (RGD) domain of FN has been termed the cell-binding domain due to its presence in several other ECM proteins known to bind to cells (Hersel et al., 2003; Pierschbacher and Ruoslahti, 1984; Ruoslahti and Pierschbacher, 1987). Although many integrins can bind FN, the $\alpha_5\beta_1$ integrin is the

main FN receptor on most cells. In addition, binding of $\alpha_5\beta_1$ to FN has been implicated as important in supporting mitogen proliferation in endothelial, epithelial, and fibroblast cells (Danen and Yamada, 2001)

While integrins themselves do not have any intrinsic catalytic signaling ability, integrin binding to ECM proteins induces integrin clustering and subsequent focal adhesion formation that affects multiple signaling pathways within cells (Danen and Yamada, 2001). Structural components such as actin fibers and cortical actin filaments also associate with focal adhesions; thereby providing a link between integrin-mediated signaling and the cytoskeleton (Vuori, 1998). Ultimately, these complexes initiate signaling cascades that lead to cytoskeleton rearrangement and integrin-mediated gene transcription (Asthagiri et al., 1999; van der Flier and Sonnenberg, 2001). Importantly, these integrin-mediated signals enforce an adhesion requirement for proliferation in the majority of normal cells (Schwartz and Assoian, 2001).

Interestingly, adhesion alone is not sufficient to maintain survival or induce proliferation (Assoian, 1997; Asthagiri et al., 2000; Danen et al., 2000; Schneller et al., 1997; Schwartz and Assoian, 2001; Vuori and Ruoslahti, 1994). Instead, signaling events induced by soluble growth factors are also essential. It is the combined effect of signaling induced by integrin-mediated adhesion and soluble growth factors that is necessary for cell viability and proliferation; thus, it is not surprising that both adhesion and soluble growth factors affect similar signaling pathways (Assoian, 1997; Danen et

al., 2000; Pu and Streuli, 2002; Roovers and Assoian, 2000; Schneller et al., 1997; Schwartz and Assoian, 2001; Vuori and Ruoslahti, 1994).

While both integrins and growth factors affect similar signaling pathways, the means by which they elicit these signaling events differ. Unlike integrins, soluble growth factors, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and insulin, do not affect the cell structure. Instead, they actively induce signaling by directly binding to their growth factor specific receptor. This ligand binding induces the catalytic activity of the transmembrane receptor and elicits a signal cascade ultimately affecting numerous cellular functions.

Because of the physiological importance of adhesion- and growth factor-mediated cell regulation, significant attention has been given to uncovering the underlying signaling mechanisms relating the two. One prominent point of crosstalk between adhesion and growth factors involves the serine/threonine kinase, extracellular signal-regulated kinase (Erk). Although Erk activation is generally viewed as a result of activation of the canonical growth factor-mediated mitogen activated protein kinase (MAPK) signaling pathway, integrin-mediated signaling ultimately results in the enhancement of Erk activation. While we leave a detailed discussion of the pathways by which integrins affect and enhance MAPK signaling to Chapter 4, growth-factor mediated activation of the canonical MAPK pathway is presented here.

Growth factor-mediated activation of the canonical MAPK pathway begins with the binding of a soluble growth factor ligand to its respective receptor. The ligand binding elicits tyrosine autophosphorylation of the receptor (Ford and Pardee, 1999; Roovers and Assoian, 2000), which enables growth factor receptor bound protein 2 (Grb2) to bind to the now activated receptor. The Grb2-growth factor receptor complex then recruits and binds with the guanosine 5'-triphosphate exchange factor, son-of-sevenless (Sos) (Roovers and Assoian, 2000). Sos-mediated nucleotide exchange activates Ras by converting it to its GTP form, which enables it to bind to and activate Raf, initiating activation of the MAPK cascade. Active Raf kinase binds to and activates Mek, which binds to and activates Erk, eventually leading to S-phase entry (Ford and Pardee, 1999; Roovers and Assoian, 2000) if also activated by integrin-mediated pathways.

In most normal cells, Erk must be activated by both integrins and growth factors in order to induce proliferation. Thus, it is the combined effect of integrin-mediated activation pathways and growth factor-induced signaling events that enables active Erk to translocate into the nucleus. Once inside the nucleus, active Erk phosphorylates Elk-1 and enables cyclin D1 transcription (Aplin et al., 2001; Ford and Pardee, 1999; Khokhlatchev et al., 1998; Pu and Streuli, 2002; Roovers and Assoian, 2000). Cyclin D1 then forms a complex with cyclin-dependent kinase 4/6 (cdk4/6) that is able to phosphorylate the tumor-suppressor retinoblastoma protein (Rb) (Assoian, 1997; Ford and Pardee, 1999; Pu and Streuli, 2002). Rb is normally complexed with E2F. However, upon phosphorylation, Rb is released from the E2F complex, enabling the transcription of

numerous genes involved in DNA synthesis and ultimately leading to S-phase entry and proliferation (Assoian, 1997; Ford and Pardee, 1999; Pu and Streuli, 2002).

The nature of Erk signaling has been intensely studied. While some studies suggest that it is the early phase of growth factor-mediated Erk signaling that is enhanced by adhesion (Danen et al., 2000; DeMali et al., 1999; Lin et al., 1997), others have suggested that it is dynamic changes in Erk activation, and not the early magnitude of the signal, that is important (Danen et al., 2000; Jones and Kazlauskas, 2001; Renshaw et al., 1999; Roovers et al., 1999; Tombes et al., 1998). Yet others still report no synergism between adhesion and soluble growth factors in Erk signaling (Hedin et al., 1997; Mettouchi et al., 2001). These discrepancies are attributable to the use of different cell systems, different synchronization methods, different growth factor cocktails, and different adhesive contexts (Danen et al., 2000; DeMali et al., 1999; Renshaw et al., 1997; Roovers et al., 1999). These discrepancies in experimental protocols make it difficult to decipher whether adhesion enhances Erk signaling in response to all of these growth factors or whether only a subset of growth factors signal in an adhesion-dependent manner. In addition, conclusions regarding differences in dynamic activation profiles (either manifesting themselves as a difference in magnitude or as a shift from transient to sustained signal) cannot be resolved from the current body of literature.

In an effort to answer these questions, we chose to examine quantitatively the effect of adhesion and specific growth factors on Erk activation. We first developed an experimental system that enables systematic comparison of the crosstalk between adhesion and individual growth factors. In addition, we developed a quantitative protocol for Western blot imaging and analysis to measure both the magnitude and dynamics of Erk signaling. The development the experimental system and quantitative assay are discussed in Chapter 2.

Interestingly, utilization of our experimental protocol reveals a novel aspect of adhesion-dependent growth factor signaling. As described in Chapter 3, we discovered that adhesion selectively desensitizes growth factor-mediated activation of Erk. To help guide our search for the molecular mechanism(s) responsible for differential Erk activation dynamics by specific growth factors, we developed a simple coarse-grain mathematical model, which is described in detail in Chapter 4. Finally, Chapter 5 outlines both the significance of our results and how model predictions of key molecular mechanisms may be further examined in future work.

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Chapter II. Developing an Experimental Platform for Quantifying Adhesion-Dependent Signaling

1. Abstract

A prominent point of signaling crosstalk between adhesion and growth factors involves the serine/threonine kinase, extracellular signal-regulated kinase (Erk). Extensive effort has been invested in understanding adhesion-dependent Erk signaling. However, because of the considerable differences in protocols and the qualitative nature of past studies, the effect of individual growth factors on adhesion-dependent Erk signaling remains unclear. To address these issues, we developed: (1) an experimental system that enables systematic comparison of the crosstalk between adhesion and individual growth factors and (2) a quantitative protocol for measuring both the magnitude and dynamics of Erk signaling. The experimental system avoids potentially confounding contributions from serum by conducting each step of the protocol in completely serum-free medium. We find that serum augmentation is necessary to maintain NIH-3T3 cell viability only when late signaling kinetics are examined. We have identified a 4-5 hour window during which serum supplementation is unnecessary to maintain cell viability. These serum-free conditions, coupled with optimized methods for maintaining cells in suspension, should now enable us to directly study the adhesion-dependence of specific growth factors. Unique to existing methods used to quantify adhesion-dependent Erk activation, we also employ standards in every Western blot to confirm the linear range of the assay. These standards are also used to optimize antibody incubation protocols. The development of a rigorous experimental system and quantitative methods for imaging and analysis well equips us to investigate the effect of

integrins and individual growth factors on adhesion-mediated signaling, which we discuss in subsequent chapters.

2. Introduction

Cell adhesion generates intracellular signals that affect multiple cellular functions including proliferation, survival, and apoptosis (Asthagiri et al., 1999; Giancotti and Ruoslahti, 1999; van der Flier and Sonnenberg, 2001; Vinogradova et al., 2002; Vuori, 1998). While proliferation of mammalian cells is dependent on a myriad of environmental factors, stimulation both by soluble growth factors and by integrin-mediated adhesion is among the most important. In fact, loss of adhesion in normal cells generally results in complete cell cycle arrest (Assoian, 1997; Schwartz and Assoian, 2001). In addition, loss of integrin-mediated cell adhesion initiates apoptosis in susceptible cell types such as endothelial human umbilical vein (HUVEC) cells (Meredith et al., 1993), gut epithelial cells (Meredith et al., 1993), normal Madin-Darby canine kidney (MDCK) epithelial cells (Frisch and Francis, 1994), and spontaneously immortalized non-transformed human keratinocyte (HaCat) cells (Frisch and Francis, 1994). Because the majority of normal cells require adhesion to an ECM in order to proliferate, it is not surprising that deregulation of adhesion-dependent proliferation often contributes to cancer development. In fact, the formation and spread of tumors is closely associated with decreased dependence on adhesion to extracellular matrix proteins (Assoian, 1997; Schwartz and Assoian, 2001).

An important integrin-mediated signaling pathway involves activation of the mitogen activated protein kinase (MAPK) pathway, which is also triggered by growth

factor stimulation (Danen et al., 2000; Ford and Pardee, 1999; Giancotti and Ruoslahti, 1999; Pu and Streuli, 2002; Renshaw et al., 1997; Roovers and Assoian, 2000; Roovers et al., 1999; Schlaepfer et al., 1994; Schneller et al., 1997; Schwartz and Assoian, 2001; Vuori and Ruoslahti, 1994). Interestingly, Erk activation by either integrins or growth factors alone is not sufficient for S-phase entry, and, by extension, proliferation (Assoian, 1997; Danen et al., 2000; Schneller et al., 1997; Schwartz and Assoian, 2001; Vuori and Ruoslahti, 1994). However, the combined effects of integrins and growth factors have been shown to synergistically amplify Erk signaling and ultimately lead to S-phase entry and proliferation (Assoian, 1997; Danen et al., 2000; Pu and Streuli, 2002; Roovers and Assoian, 2000; Schneller et al., 1997; Schwartz and Assoian, 2001; Vuori and Ruoslahti, 1994).

While some studies suggest that it is the early magnitude of Erk signaling that confers its adhesion-dependence (Danen et al., 2000; DeMali et al., 1999; Lin et al., 1997), others have suggested that it is the changes in Erk signaling dynamics over extended time periods that are actually important (Danen et al., 2000; Jones and Kazlauskas, 2001; Renshaw et al., 1999; Roovers et al., 1999; Tombes et al., 1998). Yet others report no synergism between adhesion and soluble growth factors in Erk signaling (Asthagiri and Lauffenburger, 2000; Hedin et al., 1997; Mettouchi et al., 2001). These discrepancies may be attributed to the use of different cell systems, different synchronization methods, different growth factor cocktails, and different adhesive contexts. For example, while NIH-3T3 cells are prevalent throughout the literature (Danen et al., 2000; Renshaw et al., 1997; Roovers et al., 1999), other cell type, including

Ph cells and F cells (DeMali et al., 1999) or NIH-3T3 cells expressing transfected constructs, are also used (Roovers et al., 1999). In addition, not all studies serum-starve cells before plating (Renshaw et al., 1997), and some include small amounts of serum in the starvation medium (DeMali et al., 1999; Renshaw et al., 1997). Serum is also included in the growth factor stimulation medium, with the amount of serum varying from as little as 0.4% (Renshaw et al., 1997) to as much as 5% (Roovers et al., 1999). Use of multiple growth factors in stimulation medium also occurs (Danen et al., 2000)

Furthermore, not all adhesion studies use defined surfaces, where a single ECM protein, such as fibronectin (FN), is exclusively used to mediate cell adhesion. Instead, some allow the cells to adhere to tissue culture dishes coated with ECM proteins from serum prior to growth factor stimulation (Renshaw et al., 1997; Roovers et al., 1999). The method for maintaining non-adherent cells also varies in the literature; some employ poly-L-lysine(PL)-coated surfaces to mediate non-specific attachment via electrostatic interactions (DeMali et al., 1999), while others utilize agarose-coated dishes to maintain cells in suspension (Danen et al., 2000; Renshaw et al., 1997; Roovers et al., 1999). The time cells are either maintained in suspension or allowed to adhere to FN-coated surfaces prior to growth factor stimulation, or the 'priming time,' also varies widely in the literature. Some prime cells for substantial amount of time prior to growth factor stimulation (Danen et al., 2000; Renshaw et al., 1997), while others choose not to prime cells at all (Roovers et al., 1999).

Finally, adhesion-mediated Erk signaling is often analyzed only in qualitative terms. In studies that attempt to quantify Erk signaling, densitometry of film-based techniques is often used. As film-based techniques are known to easily saturate, and standard curves are not provided with the data, one can never be certain that quantitative data obtained via densitometry occurs within the linear dynamic range of the imaging protocol.

Because of these discrepancies in experimental protocols, it remains unclear whether adhesion-dependent Erk signaling is specific to particular growth factors. Thus we sought first to establish an experimental system to study adhesion-dependent signaling, and then to develop quantitative methodologies to accurately measure these adhesion-mediated signals.

3. Results and Discussion

3.1 *Considerations in developing a protocol to study adhesion-mediated signaling*

Adhesion and growth factor synergy has been investigated by comparing differences in signaling between adherent and non-adherent cells stimulated by growth factors. However, due to the many the differences in both the experimental systems and the protocols employed in previous adhesion-dependent studies, it remains unclear whether adhesion-dependent Erk signaling is specific to particular growth factors. Thus, we are left with many possibilities for which cell type to examine, how to lift cells from the dish, which kind of adhesive and non-adhesive surface to utilize, whether starvation

medium is necessary to quiesce residual Erk signaling in the cells, and whether supplementing starvation and/or stimulation media with serum is required to maintain high cell viability. Because our goal is to develop an adhesion protocol that enables direct comparison of the effects of adhesion and a single growth factor on Erk signaling, each protocol development decision we make will be with this objective in mind.

In order to fulfill our goal of developing an experimental system to enable direct comparison of three individual growth factors (EGF, PDGF, or bFGF) on adhesion-dependent Erk activation, it is essential that each experiment be conducted using the same experimental system. Each experiment must utilize the same cell type, a consistent method to lift cells, the same plating method, identical starvation medium formulations, equivalent stimulation medium formulations prior to growth factor addition, equivalent plating methods for adherent and non-adherent cells, and identical analysis and imaging techniques. Thus, we seek to develop a consistent protocol to acquire data that will begin to provide a systematic comparison of the crosstalk between adhesion and individual growth factors.

Development of this experimental protocol required careful consideration of the effect that each choice we made would have on our experimental system. For example, during determination of the appropriate cell detachment technique, we had to consider how each option would affect the integrity of the integrins. Integrin damage is known to occur via protease exposure. Thus, we were careful to add a protease inhibitor to cells

immediately after detachment when a protease-containing cell detachment method was used.

The possible formation of cell aggregates following detachment was another important consideration. Cell aggregation must be prevented because such cell-cell contact can initiate cadherin-mediated signaling that convolutes proper data interpretation. Although some cell-cell contact during re-suspension is likely, cells can be quickly separated via trituration to limit the effects of cadherin-mediated signaling. However, excessive trituration can negatively affect cell viability, and thus should be avoided. In cases where the cell aggregates cannot be separated, the cell suspension must not be used for experiments due to probable cadherin-mediated signaling.

The presence of residual adhesion-mediated signaling resulting from cell attachment to matrix proteins in serum is an equally important consideration. Cells are cultured on uncoated tissue culture dishes in serum-containing growth media. To return Erk signaling to basal levels and prevent residual Erk activation from confounding interpretation of adhesion- and/or growth factor-induced signaling, serum-starvation prior to re-plating of cells for experiments was investigated. As quiescing residual signals by deprivation of both serum and integrin engagement is known to induce apoptosis (Levine and Klionsky, 2004; Reddig and Juliano, 2005), maintaining cell viability is also a concern. Clearly, from a data-analysis standpoint, excluding serum from starvation and stimulation media is most desirable. However, the cells must remain viable to obtain

valid results. Therefore, the balance of serum use and cell viability was closely evaluated.

In addressing these considerations, we developed an experimental protocol that limits the use of serum to ensure that signaling responses can be clearly attributed to adhesion and a specific growth factor. Moreover, conditions that minimize cell clustering and quiesce pre-stimulatory Erk signaling were identified. Together, the developed experimental protocol provides a systematic platform for parsing the crosstalk between cell adhesion- and growth factor-mediated signaling.

3.2 *Maintaining high cell viability while eliminating cell aggregation*

Our studies focused on the NIH-3T3 cell line, since it has served as the preferred line for adhesion-mediated signaling studies (Danen et al., 2000; Renshaw et al., 1997; Roovers et al., 1999). Maintaining high NIH-3T3 cell viability is a concern through each phase of the protocol, including the step where cells are detached from the culture dish. Multiple factors can affect cell viability during detachment including prolonged exposure to proteases and removal of cells via mechanical shearing. In addition to cell viability issues, the formation of cell aggregates following cell detachment can also readily occur.

A variety of solutions can be used for cell detachment, with some containing only chelating agents (such as EDTA) and others containing a combination of both chelating agents and proteases (such as trypsin diluted in EDTA). However, chelating agents alone do not always result in complete detachment. Thus, the presence of a protease can be

required to further detach the cell from the dish. However, as proteases are known to damage cell surface receptors, care must be taken to avoid prolonged protease exposure. Thus, the lowest concentration of protease necessary to induce efficient detachment should be used to mitigate potential cell-receptor damage.

We investigated six different detachment agents: 5 mM EDTA alone, 0.48 mM EDTA alone, 0.01% trypsin diluted in 5 mM EDTA, 0.01% trypsin diluted in 0.48 mM EDTA, 0.05% trypsin diluted in 0.48 mM EDTA, and 0.25% trypsin diluted in 1 mM EDTA. Although 0.48 mM EDTA and 5mM EDTA elicit the least amount of cell-receptor damage due to the absence of a protease, neither method was able to induce cell detachment even after exposure of up to 45 min. As a result, substantial mechanical shearing from the force of the EDTA solution being washed over the dish during cell collection was required to dislodge cells from the dish, and use of both concentrations of EDTA alone resulted in approximately 50% cell viability as assayed via trypan blue staining (data not shown).

While adding 0.01% trypsin to either 0.48 mM EDTA or 5 mM EDTA induced cell detachment within 7 min and enabled complete detachment of cells from the dish, significant cell aggregation resulted upon re-suspension. Only via vigorous trituration—which resulted in 20-40% viability, as assayed via trypan blue staining—could single-cell suspensions be obtained (data not shown). Although cell viability improved significantly when less trituration was employed, a single cell suspension could not be obtained. Thus detachment methods with only 0.01% trypsin were eliminated, due to

either probable cadherin-mediated signaling from cell aggregates that remained after mild trituration, or due to poor cell viability that occurred when vigorous trituration was used to separate cell aggregates.

When the trypsin concentration was increased to 0.05% trypsin in 0.48 mM EDTA or 0.25% trypsin in 1 mM EDTA, detachment again occurred within 7 min. Single-cell suspensions were obtained after mild trituration and high cell viability was maintained, establishing both as potential detachment agents. However, as trypsin is known to elicit some degree of integrin and receptor damage, the solution with the lower concentration of trypsin, 0.05% trypsin in 0.48 mM EDTA, was selected as optimal. To further minimize cell-receptor damage, we employed soybean trypsin inhibitor immediately following cell detachment. Thus, although protease use cannot be avoided, as its presence is required for both high cell viability and elimination of cell aggregate formation upon re-suspension, we efficiently mitigate its potential negative effects via low trypsin concentration, short contact time, and the addition of an inhibitor immediately following detachment.

Cell viability and aggregation issues can also occur as a result of the methodologies used for either facilitating or preventing cell adhesion. While undefined surfaces coated with ECM proteins found in serum can be used to facilitate adhesion (Renshaw et al., 1997; Roovers et al., 1999), it is difficult to ascertain the contribution of specific integrins on signal activation. Thus, we used a defined surface to facilitate adhesion; specifically, we selected fibronectin, as it is the most common ECM used in

defined surface studies (DeMali et al., 1999; Lin et al., 1997; Renshaw et al., 1997; Roovers et al., 1999).

To complement the experimental condition where cells are adhered to a fibronectin-coated substratum, a condition where cells are cultured in an adhesion-free environment was also developed. A survey of the literature revealed that methods for preventing integrin-mediated attachment vary widely. While some studies use poly-L-lysine (PL)-coated surfaces to prevent adhesion-mediated attachment due to integrin binding (DeMali et al., 1999), others use agarose-coated tissue culture dishes to maintain cells in suspension (Danen et al., 2000; Renshaw et al., 1997; Roovers et al., 1999). We also explored poly-HEMA (PH)-coated surfaces as a potential suspension method. Although not traditionally used in integrin and growth factor signaling studies, PH is the most widely utilized approach for holding cells in suspension during suspension-induced anoikis studies (Folkman and Moscona, 1978b; Frisch and Francis, 1994; Kawada et al., 1997; Lebowitz et al., 1997; Reginato et al., 2003). The mechanism by which adhesion is inhibited via each of these methods is further discussed in the following paragraphs.

PL enables cell attachment to the culture dish via an electrostatic interaction between the cell membrane and the tissue culture dish surface. Thus, although no integrin engagement should occur when cell are adhered to PL, cells do experience an electrostatic force that holds them to PL-coated surface. We eliminated PL as the optimal method for non-adherent cells because the cells are subjected to an electrostatic force absent in the adherent condition.

Agarose-coated dishes inhibit cell attachment by presenting a surface that cells supposedly cannot adhere to. However, preliminary experiments using agarose-coated dishes that were gently rocked to avoid the settling of cells proved this method unacceptable for use as substantial cell aggregation resulted (Figure II-1A). Moreover, cells embedded into the agarose surface over time, resulting in poor cell recovery. Additionally, actual cell adhesion to the agarose coated surface also occurred (Figure II-1A). Clearly, agarose is not an ideal surface for the suspension protocol.

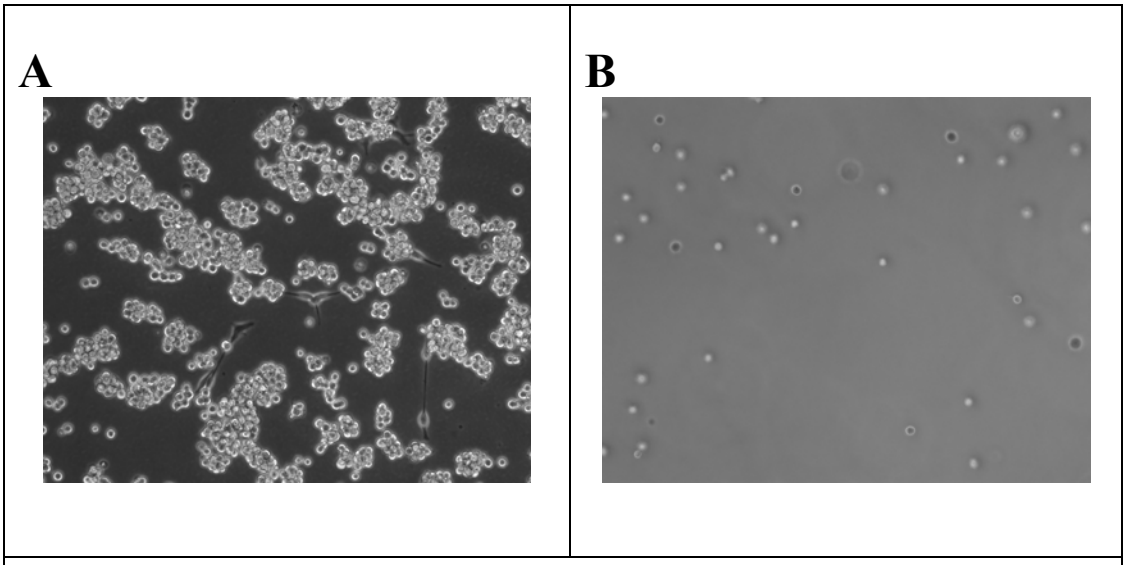

Figure II-1: Suspension on poly-HEMA-coated dishes
NIH-3T3 cells were starved for 20 h, allowed to adhere for 2.5 h, and stimulated with 0.81 mM of PDGF. **(A) Severe cell aggregation occurs on agarose-coated dishes** Cells plated on 60 mm agarose dishes containing 3 mL total medium volume experience substantial clumping. Some adhesion to the agarose-coated surface is also observed. **(B) PH-coated dishes eliminate cell clumping** Cells plated on 100 mm PH-coated dishes containing 11 mL total medium volume subjected to gentle rocking displays no cell aggregation.

As mentioned previously, PH is the most commonly used substrate for holding cells in suspension during anoikis studies. When alcoholic solutions of PH are allowed to evaporate onto a plastic tissue culture dish, a thin, hard, non-ionic sterile film of optically clear polymer remains tightly bonded to the plastic surface. This non-ionic film inhibits cell attachment by preventing matrix deposition and subsequent cell adhesion to the PH-coated surface (Folkman and Moscona, 1978a; Frisch and Francis, 1994). Although during initial testing, cells plated on PH-coated dishes settled to the bottom of the dishes and cell aggregates formed, increasing the total medium volume from 3 mL in a 60 mm dish to 11 mL in a 100 mm dish and subjecting the dishes to gentle rocking eliminated these problems (Figure II-1B).

3.3 *Maintaining high cell viability while minimizing serum supplementation*

Maintaining high cell viability has been an important issue during the development of our experimental protocol. Although minimizing serum use is important in determining the contribution of individual growth factors on signal activation, the decision of whether or not to use serum in the starvation and/or stimulation media completely relies upon whether or not serum is necessary to sustain high cell viability.

NIH-3T3 cells undergo apoptosis as a result of prolonged exposure to completely serum-free medium (data not shown). As mentioned previously, serum augmentation of the starvation and stimulation media improves cell viability, but its presence also prevents ascertainment of the effect of individual growth factors on Erk activation due to the presence of the many signal-inducing proteins present in serum. Moreover,

restoration of basal signal activation levels is unattainable in the presence of large amounts of serum. For these reasons, exclusion of serum from the experimental protocol is ideal.

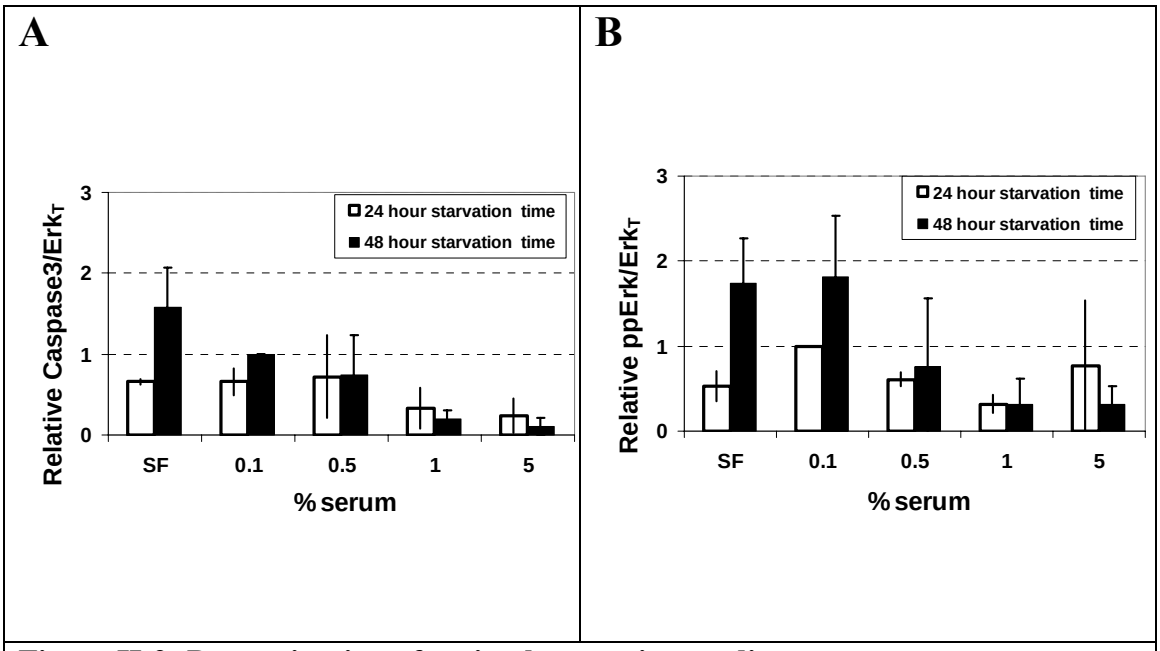

Figure II-2: Determination of optimal starvation medium.
NIH-3T3 cells were starved for 24 h (empty) or 48 h (filled) in completely serum-free medium (serum-free) or in completely serum-free medium supplemented with increasing amounts of serum (0.1% serum, 0.5% serum, 1% serum, or 5% serum). All proteins have been normalized to their respective total Erk equal loading control. **(A) Measure of apoptosis resulting from starvation** Normalized cleaved caspase3 induction after 24 h (white bars) or 48 h (black bars) of starvation. **(B) Ability of starvation media to quiesce Erk Activation** Normalized Erk activation after 24 h (white bars) or 48 h (black bars) of starvation.

To determine if the presence of serum was necessary for high cell viability during quiescing of the cells, the presence of cleaved caspase 3, a known marker of apoptosis, was assayed for cells exposed to starvation medium containing increasing concentrations of serum. Cells were starved for 24 h and 48 h in completely serum-free medium or in completely serum-free medium supplemented by 0.1%, 0.5%, 1%, or 5% serum. After

24 h, there was little difference in survival of cells starved in completely serum-free medium as compared to cells starved in serum-free media supplemented with either 0.1% or 0.5% serum (Figure II-2A). Supplementation by 1% and 5% serum reduced cleaved caspase3 to nearly basal levels. Starvation in a completely serum-free medium for 48 h was not an option, as NIH-3T3 cells are unable to survive serum deprivation for such an extended amount of time. After 48 h, the majority of the cells had detached from the dish (observations) and a significant portion of the remaining cells on the dish were undergoing apoptosis. Interestingly, very little difference in cell survival was observed between 24 h of starvation verses 48 h of starvation for 0.1%, 0.5%, 1%, and 5% serum-supplemented serum-free media (Figure II-2A).

The optimal starvation condition must minimize apoptosis and return Erk signaling to basal levels. Thus, we also examined the ability of each starvation condition to quiesce Erk signaling. Recall that quiescing cells in a completely serum-free medium to return adhesion signals to basal levels is ideal, as it enables interpretation of individual growth factors without the presence of serum to convolute the results. Although starvation in completely serum-free medium after 24 h quiesced Erk signaling to basal (near zero) signal levels (Figure II-2B), the presence of small amounts of cleaved caspase3 (Figure II-2A) indicate that a small fraction of cells are undergoing apoptosis. Cells starved for 48 h in medium supplemented with either 1% or 5% serum were also able to quiesce Erk activation back to basal (near zero) signal levels (Figure II-2B) and these were the most effective starvation conditions in minimizing the induction of

cleaved caspase 3 (Figure II-2A). However, the presence of such large amounts of serum in the starvation medium convolutes interpretation of the data.

As we suspected, the decision of whether or not to augment the medium with serum was a balance between quiescing Erk signaling and maintaining high cell survival. While the two starvation media with the highest levels of serum are optimal for viability (Figure II-2A), they are the worst conditions from a data-interpretation perspective. Likewise, while elimination of serum is the best choice for data interpretation, completely serum-free medium induces the highest levels of apoptosis. Thus, no acceptable combination of serum and viability could be found for starvation times of 24 h or 48 h.

We hypothesized that by reducing the starvation time of the completely serum-free condition, we might find an optimal starvation time where low caspase3 induction and effective quiescing of basal signaling resulted from the completely serum-free medium condition. We found that when the starvation time is reduced to 20 hours, caspase 3 induction remains low, and both Erk and Akt signaling are quiesced (data not shown). Thus, starvation in completely serum-free medium was selected as the optimal choice for quiescing basal signaling in the cell, with experimental starvation times not to exceed 20 h.

The compromise between cell viability and clear data interpretation also arises in the stimulation medium formation. The duration of adhesion/suspension time prior to growth factor stimulation, or the priming time, is a variable of interest in our

experiments. Thus, we had to determine how long cells could be deprived of growth factor stimulation before cell viability becomes an issue, with and without serum augmentation.

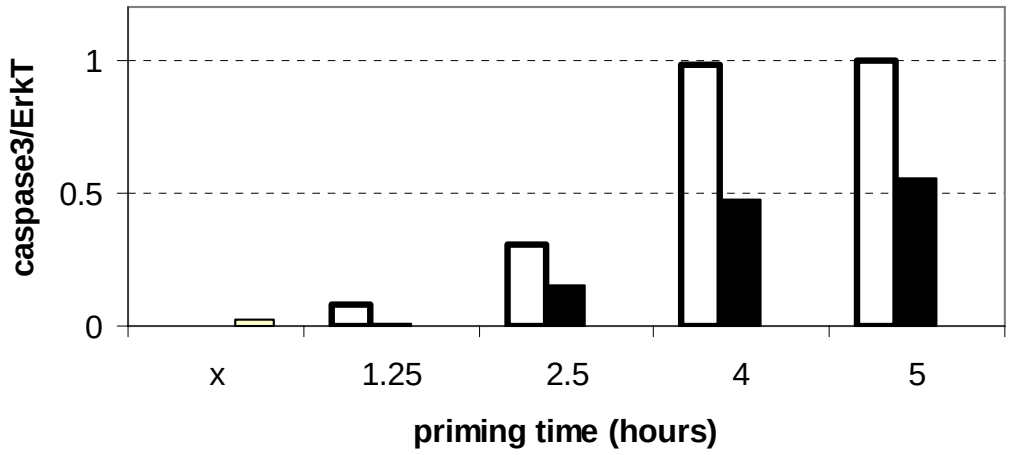

Figure II-3: Induction of cell apoptosis as a function of time in suspension
NIH-3T3 cells were starved for 20 h in completely serum-free medium, plated on FN-coated dishes (adherent cells, black bars) or maintained in suspension via PH-coated dishes (suspended cells, white bars) for increasing amounts of time. The unstimulated cells were assayed for the presence of cleaved caspase 3 at different priming times, and cleaved caspase 3 normalized to its total Erk equal loading control is shown. "X" denotes cells prior to plating (that have been starved for 20 h).

Cells starved in completely serum-free medium for 20 h, were plated on either FN- or PH-coated surfaces for various lengths of time to assay cell viability as a function of priming time (Figure II-3). We find that cleaved caspase 3 induction remains low both prior to plating and after priming times for 1.25 h on either FN- or PH-coated surfaces. By 2.5 h, caspase 3 induction has increased slightly and by 4 h caspase 3 induction is significant, indicating that considerable apoptosis has occurred. This data reveals that

cells can be serum-starved for 20 h and primed for up to 2.5 h without serum augmentation.

To establish whether or not serum supplementation of the stimulation medium was necessary to sustain high cell viability, the presence of cleaved caspase 3 was assayed after cells were starved for 20 h and primed for 2.5 h in completely serum-free media, as these are the harshest starvation and priming times conditions found to support high cell viability. Caspase 3 induction over a period of two hours after stimulation by EGF, bFGF, or PDGF remained either at or below basal signal induction (data not shown). Thus, serum supplementation of the stimulation medium is not required to study Erk signaling kinetics for priming times up to 2.5 hours and for stimulation times up to 2 hours (data not shown).

Consequently, if the effects of adhesion-dependent signaling for priming times longer than 2.5 hours or if proliferative studies requiring the negative control of unstimulated cells are to be studied, serum supplementation of both the starvation and stimulation media are necessary to enhance cell viability. In accordance with these findings, cell cycle progression studies supplement the starvation and stimulation media with serum (Renshaw et al., 1997; Roovers et al., 1999). However, for the purposes of our investigation, which investigates early Erk signaling kinetics, serum supplementation is not necessary.

3.4 *Summary of optimized protocol*

Employing this optimized protocol (Figure II-4), adhesion and spreading were excellent (Figure II-5A) as compared to cell adhesion and cell spreading from cells

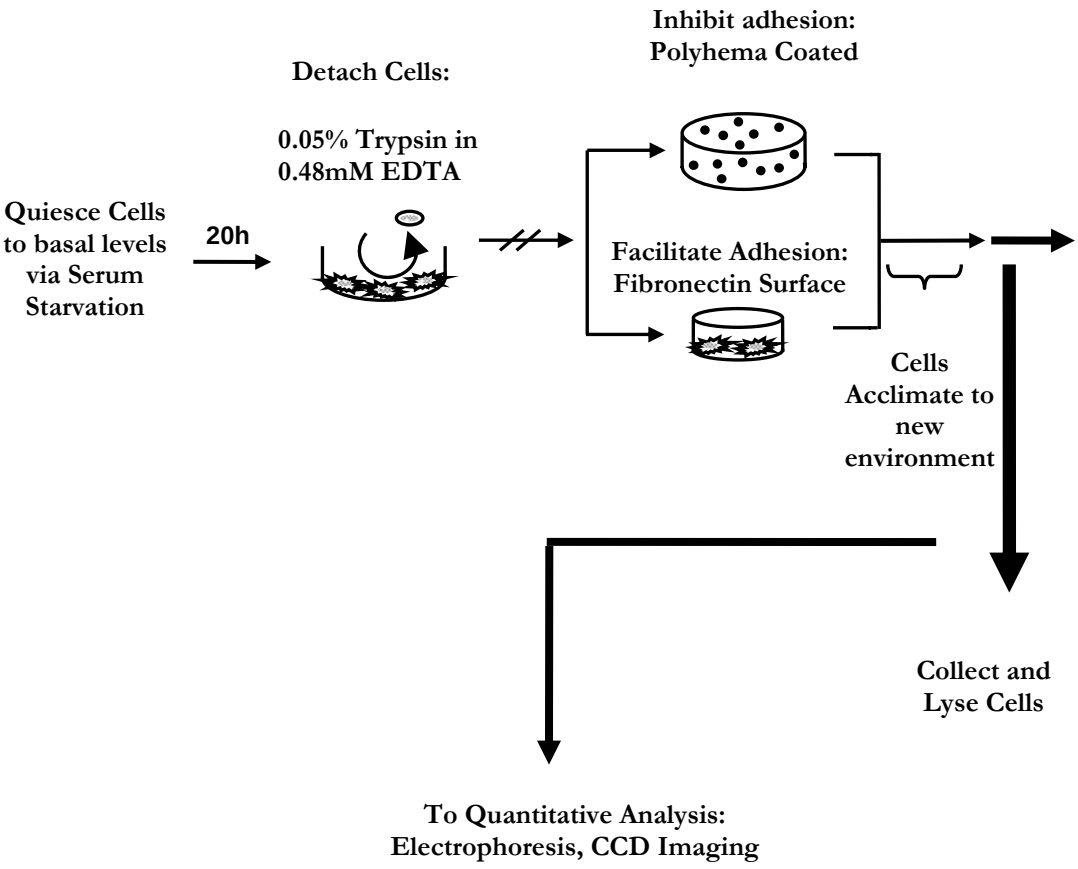

Figure II-4: Optimized adhesion protocol
Adhesive signals in subconfluent NIH-3T3 cells are quiesced by starvation for 20 h using completely serum-free medium. Cells are detached via 0.05% trypsin in 0.48 mM EDTA and re-suspended to a concentration of $5*10^5$ cells/mL. $5*10^5$ cells are plated on either FN- or PH- coated dishes. After acclimating to their new environment for a prescribed amount of time, cells are stimulated by a single growth factor and lysed at the appropriate time.

subjected to a non-optimal protocol (Figure II-5B). Cells from the non-optimal experimental protocol completely failed to adhere and spread, while cells from the optimal protocol adhered and spread with a morphology consistent with normal growth.

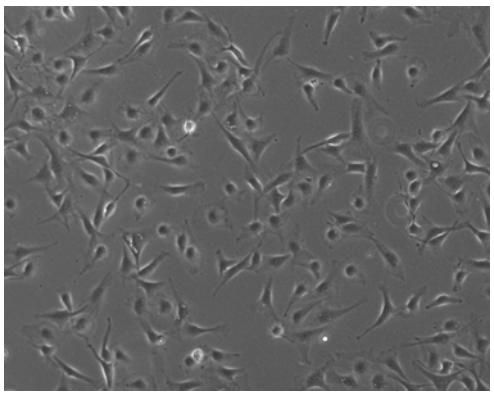

Figure II-5: Adherent NIH-3T3 cells using different adhesion protocols
NIH-3T3 cells were starved and allowed to adhere for 2.5 h before growth factor stimulation. (A) Adherent cells starved for 20 h and treated using the optimized protocol. (B) Adherent cells starved for 24 h and treated using sub-optimal DMEM (see Materials & Methods).

3.5 *Developing a quantitative methodology for measuring Erk signaling*

The next set of tools needed were analysis techniques to facilitate measurement of the lysates generated in our optimized experimental protocol. To quantify the extent to which different growth factors induce adhesion-dependent Erk signaling, we developed a systematic, quantitative, Western blotting protocol. The methodology is based on digital imaging using a cooled CCD camera that has a theoretically wider linear dynamic range than standard film-based imaging (Budowle et al., 2001; Martin and Bronstein, 1994). However, even when signals are within the detection limit of the imaging system, data

points do not always conform to the expected linear trend (Figure II-6). We expect that factors such as antigen saturation may contribute to the observed non-linearity.

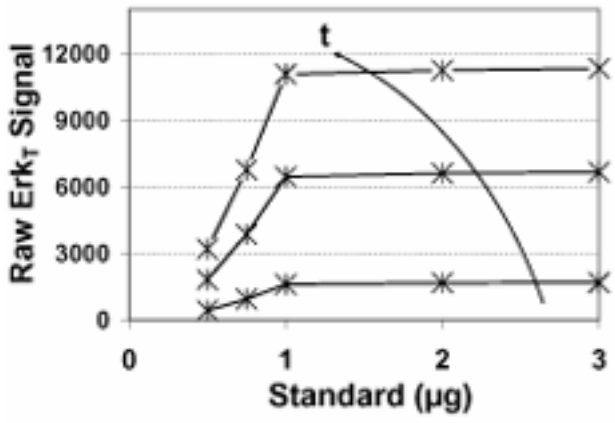

Figure II-6: Blot saturation occurs even when operating within the imaging system detection limit
Although signal strength increases with increasing image integration time, blot saturation exists for all three integration times. Thus, blot saturation cannot be eliminated by changing the integration time.

To address these sources of non-linearity that may be specific to each blot, we developed a quantitative Western blotting protocol that employs standard samples to establish the linear dynamic range of each blot. The standards are a set of dilutions of a positive control lysate as illustrated for an anti-Erk Western blot in Figure II-7A. The band intensities from the standard lanes are quantified, and the working linear range is established empirically for each blot (Figure II-7C). Band intensities from the lanes loaded with lysates-of-interest are then confirmed to fall within the linear dynamic range (Figure II-7C); any band intensities that fall outside the linear dynamic range are discarded. The sole exceptions to this requirement are samples whose basal signal cannot

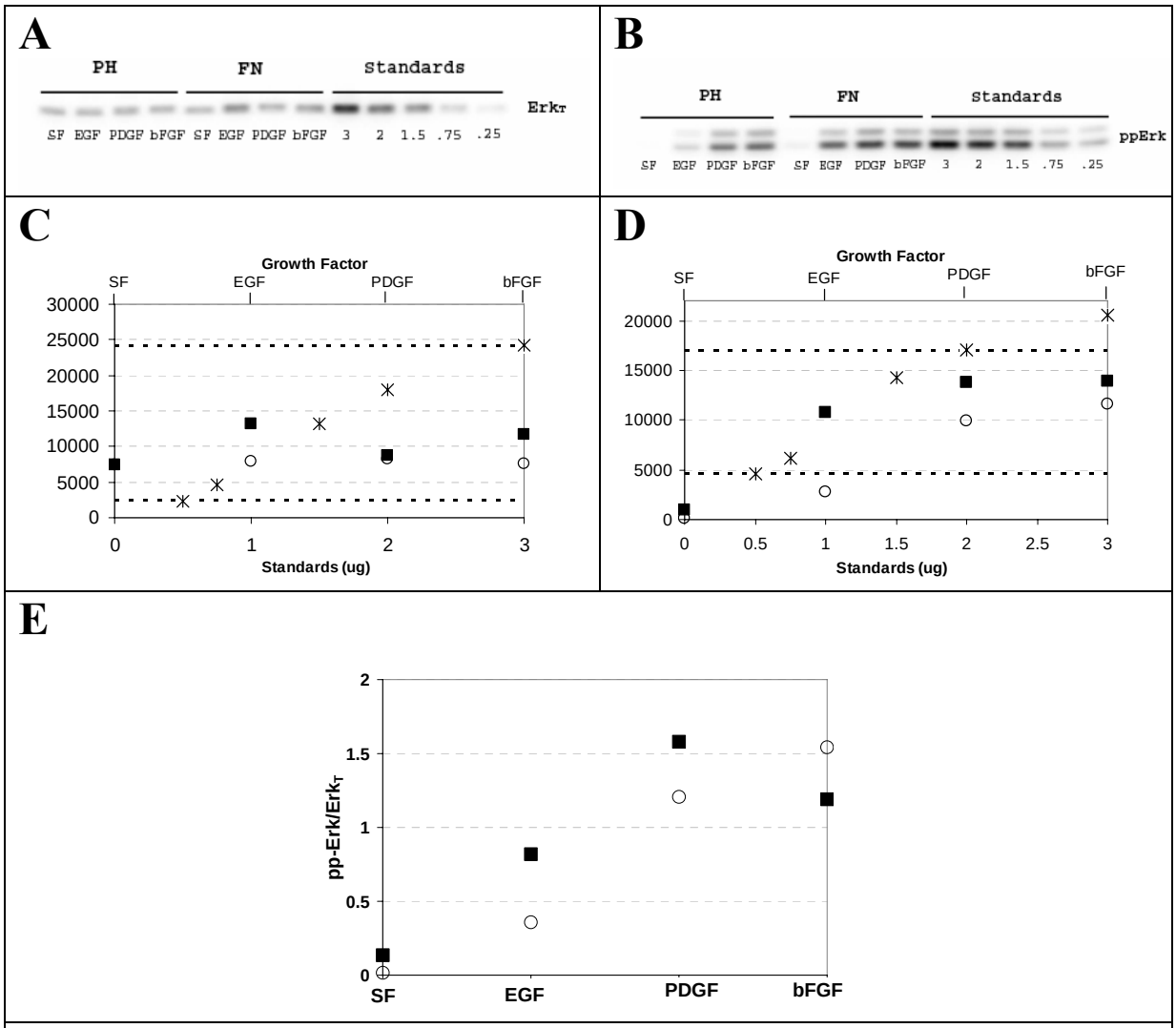

Figure II-7: Quantification of Western blotting Images
Immunoblot obtained using cooled CCD camera are shown in **(A)** for Erk_T and **(B)** for ppErk. Lanes 1-4 in each blot represents cells held in suspension for 2.5 hr, prior to stimulation for 12 min by one of four different growth factors. Lanes 5-8 represent cells allowed to adhere to FN for 2.5 hr prior to stimulation for 12 min by one of four growth factors. Lanes 9-13 represent standards employed in each blot to ensure that each unknown sample is within the linear dynamic range of the blot and that blot saturation has not occurred. Each of the unknown samples (lanes 1-8) and the Erk_T standards (lanes 9-13) are quantified. **(C)** Quantification of Erk_T signal for cells held in suspension on PH (open circles), cells adhered to FN (solid squares) and for each of the employed Erk_T standards (asterisks). **(D)** Quantification of ppErk for cells maintained in suspension via PH (open circles), cells adhered to FN (closed squares), and for each of the employed pp-Erk standards (asterisks). **(E)** Graph represents pp-Erk activation profile (shown in D) normalized to the total Erk equal loading control (shown in C). Because each signal is normalized by the total protein loaded, this method ensures the most rigorous means of comparing each growth factor's ability to activate Erk.

be distinguished from background noise; typical examples are the initial time-point following serum-starvation, or time-points measured after a signal has returned to basal. This approach ensures that the measurements of Erk expression levels lie within the linear dynamic range of each Western blot. A similar approach is applied to quantify phospho-Erk (ppErk) (Shown in Figure II-7B and Figure II-7D) and phospho-Akt (pAkt) (data not shown). The band intensity associated with a phospho-protein (e.g., ppErk, Figure II-7D) is normalized to the band intensity of an equal-loading control, such as total Erk (Erk_T, Figure II-7C), to adjust for unequal loading, as shown in Figure II-7E.

We note that the same standards are not used in every blot. Rather, dilutions of positive control lysates are used as standards. This approach ensures that for a particular blot, the band intensities of the standards will encompass nearly the entire linear range of the blot. Such an approach increases likelihood that the band intensities of the lysates-of-interest will fall within the linear range.

To ensure that data collected from two distinct blots can be compared to each another, we include a common reference point in each blot. This reference is a sample generated under the same stimulation conditions. The data (Figure II-7E) is then always analyzed and reported relative to this reference point, which in this case was chosen to be the PH 12 min time-point stimulated with 800 mM PDGF. The new graph resulting from normalization by the reference point is shown in Figure II-8A. Including such a reference enables comparison of repeated experiments, as shown in Figure II-8B. Note that the normalized signals (Figure II-8A) can only be compared with data that has been

normalized by an identical reference point. Thus the results of Figure II-8A can only be directly compared to another graph that has also been normalized by the PH 12 min time-point stimulated by 800 mM PDGF. Graphs without identical normalization time-points cannot be directly compared.

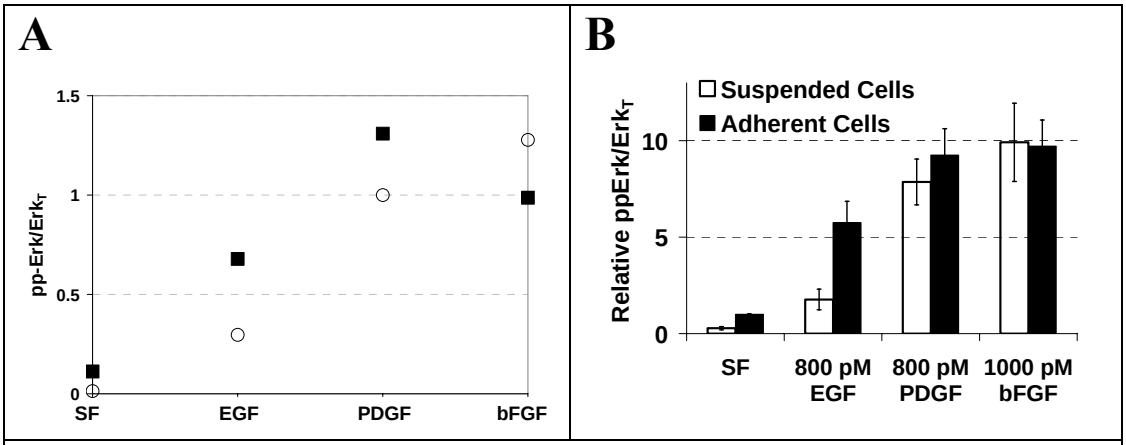

Figure II-8: Normalizing and combining data from different trials
(A) Each data point in Figure II-7E is re-normalized to the 12 min time-point for suspended cells stimulated via 800 mM PDGF so that experiments from different trials can be compared to each other. **(B)** An example of how several sets of data are normalized to the same condition (in this case, 800 mM PDGF for suspended cells) so that experiments from several different trials can be averaged together and error bars computed.

Note, however, that ratio of any two time-points in Figure II-8A can be compared to the ratio of those same time-points (provided that these two time-points are from the same second graph). For example, we can compare the ratio of the 12 min FN time-point stimulated by EGF to the 12 min PH time-point stimulated by EGF in Figure II-8A to the ratio of the 12 min FN time-point stimulated by EGF to the 12 min PH time-point stimulated by EGF in any other figure; if our data is consistent, we would find these two ratios to be identical. However, we are not able to directly compare any two equivalent time-points; for example, we cannot directly compare the 12 min FN time-point stimulated by EGF in Figure II-8A to the same time-point (12 min FN time-point

stimulated by EGF) in another figure unless both graphs have been normalized to the identical time-points (for example, 12 min PH time-point stimulated by 800mM PDGF) as described in the previously.

In order for quantitative data to correlate directly to a difference in the imaged signals, the data must fall within the linear detection range of both the blot/antibodies of interest and the cooled CCD camera itself. If data taken falls outside of the linear measurement range of either, a direct correlation between two protein band images and their relative signal strengths cannot be made. Thus, we must ensure that every blot imaged falls within both the linear dynamic range of the cooled CCD camera and the linear dynamic range of the antibody and individual blot.

Fortunately, losing data due to the occurrence of machine saturation can be readily avoided by ensuring that the feature that indicates when the camera pixel detection saturation has occurred is turned on prior to imaging the blot. This feature highlights bands where image saturation occurs; if saturation has occurred in any of the samples due to image acquisition over too long of a time period, a new image integrated for a shortened length of time can easily be acquired. It is because saturation occurring due to blot/antibody imaging cannot be eliminated during the imaging process that we invested extensive time into optimization of each antibody. By ensuring that the optimal lysate amount is loaded into every blot, and optimal antibody incubation times and incubation concentrations are employed during our Western blotting protocol, we mitigate the number of blots that must be rerun due to blot/antibody saturation. In

addition, we employ standards in every blot to provide a metric for detection of blot saturation after imaging; such a rigorous technique facilitates early detection of blot saturation so that all data falling outside the linear range can be discarded and rerun using smaller amounts of lysate.

3.6 *Extending quantitative approach to other intracellular signals*

In addition to measuring Erk signaling, protocols were developed for quantitative measurement of other signaling proteins in cell lysates. The quantitative imaging techniques utilized in this project directly affect our methodology for optimizing antibody incubation protocols. To ensure that a direct correlation exists between the observed protein band image and the actual amount of protein present in our sample, we must ensure that each lysate of interest falls within the linear dynamic range of the antibody. In order to determine the linear dynamic range of each antibody, several identical standard curves are incubated in different concentrations of the same antibody of interest, and the antibody concentration yielding the best linear dynamic range with a good signal-to-noise ratio is chosen as optimal.

For example, Figure II-9 below illustrates the process of optimizing antibody usage for the detection of phospho-Mek^{298} (p-Mek^{298}). We were originally interested in developing an optimized protocol to image phospho-Mek^{298} because of reports that phosphorylation of the serine-298 residue of Mek1 by adhesion is necessary for efficient growth factor-mediated activation of Mek, and subsequent activation of Erk (Slack-Davis et al., 2003). In order to optimize phospho-Mek^{298} for imaging, we analyzed standard curves at several different primary antibody incubation concentrations, and quantitatively

determined that a dilution of 1:5000 yielded the largest linear range between the measured signal strength and the lysate amount (Figure II-9). Moreover, a lysate amount of 7.5 µg was determined as the optimal amount to load for p-Mek^{298} imaging, as this value yielded both a high signal-to-noise ratio, and allowed for both higher and lower signal measurements to fall within the linear dynamic range of the antibody. A similar procedure was followed for each of the other antibodies of interest: pp-Erk, Erk, p-Akt, Sos, cyclin D1, and caspase 3.

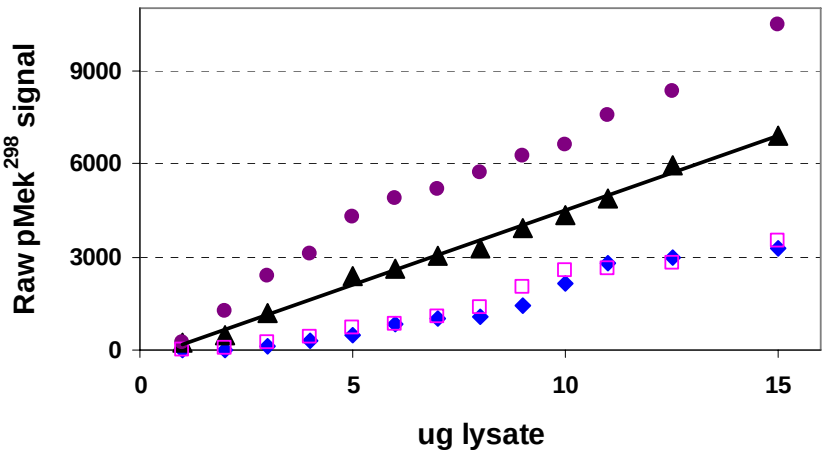

Figure II-9: Quantitative determination of optimal primary antibody conditions Four standard curve were subjected to different primary antibody dilutions of 1:1000 (solid circles), 1:5000 (Solid triangles), 1:10000 (open squares), and 1:20000 (solid diamonds).

Antibody incubation times were varied to yield optimal imaging conditions. Although the antibody incubation protocol for film exposure indicates that primary antibody incubations can be run for 1 h at room temperature or overnight at 4 ºC with equivalent results, these two conditions yielded distinctly different images when a cooled CCD camera was used to image and analyze blots. Several combinations of primary and secondary antibody incubation times were tested to optimize the obtained signal to noise

ratio for each antibody run at optimal dilution. As two optimal antibody incubation time protocols exist for film-based techniques, one which optimized for short incubation times and one optimized for long incubation times, we wanted to develop similar optimal incubation time protocols for our quantitative Western blotting imaging protocol. The results from our optimization of antibody incubation times are depicted in Table II-1 and the experimental details can be found in the material and methods section of this text.

Company	Antibody	Animal	Cat #	µg	Primary Antibodies	substrate
Biosource	pMek298	Rabbit	44-460	7.5	1:5000 ON @ 4 ºC in 3% milk/TBST or 1.25h RT, 5-10 h 4 ºC	Pico
Cell Signaling	ppErk	Rabbit	9101	1.25	1:20000 1h @ RT in 1% BSA/TBST or 1.25h RT, 5-10 h 4 ºC	Pico
Santa Cruz	Erk2	Rabbit	sc 154	1.25	1:10000 1h @ RT in 1% BSA/TBST or 1.25h RT, 5-10 h 4 ºC	Pico
Cell Signaling	pAkt	Rabbit	9271	2.5	1:1000 1h @ RT in 1% BSA/TBST or 1.25h RT, 5-10 h 4 ºC	Pico
NeoMarkers	cyclin D1	Rabbit	RM-9104	10	1:5000 ON @ 4 ºC in 1% BSA/TBST or 1.25h RT, 5-10 h 4 ºC	Pico
Cell Signaling	caspase3	Rabbit	9661	15	1:500 ON @ 4 ºC in 1% BSA/TSBT or 1.25h RT, 5-10 h 4 ºC	Pico
Santa Cruz	Sos1	Rabbit	SC-256	10	1:1000 ON @ 4 ºC in 3% milk\/TBST or 1.25h RT, 5-10 h 4 ºC	Pico

Table II-1: **Optimized antibody conditions**

The blocking buffer (1% BSA in TBST or 3% milk in TBST) is identical to that used for primary antibody incubations. Blocking is done either at room temperature (for 1h – 4h) or overnight at 4 ºC (for up to 24 h). Secondary antibodies are diluted 1:20000 in 3% milk/TBST and are incubated for either 1.5 h at room temperature or 1.25 h at room temperature and 5-8 h at 4 ºC.

4. Materials and Methods

4.1 *Cell maintenance*

NIH-3T3 fibroblasts (ATTC) were cultured and maintained in 89% DMEM (Gibco 11965-118), 10% DCS + Fe (Gibco 10371-029), and 1% PenStrep (Gibco 15070-

063). Cells were maintained under subconfluent conditions to avoid transformation, as suggested by the commercial provider (ATCC). Liquid DMEM (Gibco) was used in all cell culture, since powdered medium yielded sub-optimal performance, both in terms of cell viability and cell adhesiveness. After approximately 48 h of growth (when cells achieved 70-80% confluence) 1 mL of 0.25% trypsin/1mM EDTA (Gibco 25200-072) was added to subconfluent cells and cells were allowed to incubate at 37 ºC under 5% CO_2 for 3 min. Cells were triturated to inhibit clumping, and added to a 15 mL conical tube containing 1 mL culture media. Cells were again triturated to inhibit clumping, and were plated at a ratio of 1:5. Prior to passing, the surface of each dish was scanned twice to ensure that there were no confluent areas on the dish as NIH-3T3 are known to transform if grown to too high a confluence.

4.2 *Protein-coating surfaces*

Fibronectin (FN) (Sigma F0895) was diluted in Dulbecco's phosphate buffered saline (dPBS) (Sigma D5652) to a concentration of 5 ug/mL. 2 mL were added to each 60 mm Corning plastic tissue culture dish and the cells were incubated overnight at 4 ºC on a rocker. The dishes were then washed twice with cold dPBS and blocked with 1mg/mL filter sterilized, heat-inactivated, Bovine serum albumin (BSA A7906) diluted in dPBS for 1 h at 37 ºC under 5% CO_2. The dishes were washed twice with warm dPBS immediately before plating adherent cells.

PH (Sigma P3932) was dissolved in 70% biological grade ethanol (Sigma E7148) to a concentration of 6 mg/mL, and 5 mL was added to each 100 mm Corning plastic

tissue culture dish. The dishes were left uncovered, overnight, at room temperature, in the tissue culture hood, with the UV light off. The hood must remain slightly open to allow some air flow to occur or the ethanol will not evaporate and the PH polymer layer will not form. PH-coated dishes were then washed twice with warm dPBS prior to plating non-adherent cells.

4.3 *Cell adhesion experiments*

NIH-3T3 cells were plated at a density of 1:5 in culture media, grown for approximately 48 hours, and subconfluent dishes were starved in completely serum-free medium (99 % DMEM (Gibco 11965-118), 1% PenStrep (Gibco 15070-063), 1mg/mL BSA (Sigma A7906)) for 20 h to quiesce adhesion-mediated signaling to basal levels. Serum-starved cells were detached by adding 1mL 0.05% Trypsin in 0.48 EDTA (Gibco 25300-062) per dish, and allowed to incubate at 37 ºC under 5% CO_2 for 7 min. Cells were collected and added to a tube containing an equal volume of 1mg/mL Soybean Trypsin Inhibitor (Sigma 93619) in serum-free medium. Cells were centrifuged at 214.6 RCF for 3 min and washed twice with 3 mL serum-free medium, being careful not to agitate the cells. Cells were re-suspended in serum-free medium to a concentration of 5 x 10^5 cells/mL, and were either plated onto FN-coated 60 mm dishes containing 1 mL serum-free medium or 100mm PH-coated dishes containing 9 mL serum-free medium. Cells were incubated at 37 ºC under 5% CO_2 for a prescribed period of time (a priming time of either 1 h or 2.5 h) or no time at all (priming time of 0 h); FN-coated dishes were placed on an incubator rack, while PH-coated dishes were placed in the incubator on a rocker and gently rocked. Growth factor stimulation was achieved by adding 1 mL of growth factor stimulation medium (serum-free medium supplemented with EGF, PDGF,

or bFGF, as described in the text) to each dish so that concentration of growth factor was equivalent in both adherent and suspended dishes. FN-coated and PH-coated dishes were then incubated as described above.

After being allowed to acclimate (or prime) to their new surface for varying lengths of time, as described in the text, cells were stimulated with the indicated amount of EGF (Peprotech 100-15), PDGF (Sigma P4306), or bFGF (Sigma F0291). At desired times, Fn-coated dishes were washed twice with cold dPBS, and dishes were tilted and rested on ice for 1 to 2 minutes to allow dPBS to collect. The collected dPBS is then aspirated away. This procedure minimized the amount of diluting the cell lysate, thereby enhancing the protein concentration of the recovered lysate. Cells were lysed by adding 55 μL of lysis buffer [50 mM Tris (pH 7.5), 150 mM sodium chloride, 50 mM β-glycerophosphate (pH 7.3), 10 mM sodium pyrophosphate, 30 mM sodium fluoride, 1% Triton X-100, 1 mM benzamidine, 2 mM EGTA, 100 μM sodium orthovanadate, 1 mM dithiothreitol, 10 μg/mL aprotinin, 10 μg/mL luepeptin, 1 μg/mL pepstatin, and 1 mM PMSF] to each dish. Lysis buffer was added by touching the pipette tip to the upper surface of the dish and moving in a horizontal line as lysis buffer was added drop-wise. This procedure minimized the volume of lysis buffer needed to obtain full coverage of the 60 mm dish and contributed to higher protein concentration in the recovered lysates. The cells (on FN-coated dishes) were scraped into the lysis buffer and allowed to lyse for approximately 15 min.

For PH-coated dishes, cells were collected and centrifuged at 214.6 RCF for 2 min and liquid was aspirated. Cells were washed twice with 10 mL cold dPBS. The tube was placed on ice for 1-2 min to allow extra dPBS to collect. Most of the excess dPBS is then aspirated away and the very last bit was manually removed with a pipette. 50 μL of lysis buffer was added to each tube, cells were triturated in the lysis buffer, and allowed to lyse identically to cells on FN-coated dishes.

Lysates were centrifuged at 16060 RCF for 10 min, and the supernatant was collected using gel-loading tips to exclude residual cell debris in the suspension. Micro-BCA protein determination (Pierce 23235) was used to determine total protein concentration.

4.4 *Immunoblotting*

Whole cell lysates were resolved in 10% SDS-polyacrylamide gel electrophoresis and blotted onto a PVDF membrane. Blots were probed using either an antibody against dually phosphorylated Erk (Cell Signaling 9101L), phosphorylated Akt (Cell Signaling 9271L), or Erk2 (Santa Cruz SC-154). To assay Sos (Santa Cruz SC-256) hyperphosphorylation, a 7% polyacrylamide gel electrophoresis was used for enhanced resolution of high-molecular-weight molecules. To assay caspase 3 (Upstate 06-529), a 15% polyacrylamide gel electrophoresis was used for enhanced resolution of low-molecular-weight molecules. Blots were imaged and quantified as described in the Results and Discussion section.

4.5 *Antibody incubation time optimization*

We developed two antibody incubation protocols: one for short antibody incubation times, and one for long antibody incubation times. For optimization of the long antibody incubation time protocol, we investigated four different incubation conditions:

1. primary for 1h at room temperature (RT); secondary for 1 h at RT
2. primary overnight at 4 ºC, secondary 1 h at RT
3. primary overnight at 4 ºC, secondary overnight at 4 ºC
4. primary ~24 h at 4 ºC, secondary ~24 h at 4 ºC.

Results from this test revealed a significant difference in imaging based on the time and temperature of the primary and secondary antibody incubations. The highest signal-to-noise ratio and best linearity of the employed standard curves occurred for primary and secondary incubations that occurred overnight at 4 ºC. Following this optimized incubation protocol resulted in significantly reducing the number of blots that had to be rerun, either due to poor estimation of image acquisition time or low signal-to-noise ratio.

We then began development of an optimal short antibody incubation time protocol. Our hope was to be able to develop a protocol requiring shorter antibody incubation times that would yield equivalent results to our long antibody incubation time protocol. In order to achieve this goal, we experimented with many different combinations of antibody incubation times including:

1. primary antibody for 2 h at RT; secondary antibody for 2 h at RT

2. primary antibody for 1.5 h at RT; secondary antibody for 1.5 h at RT
3. primary antibody for 1.25 h at RT; secondary antibody for 1.25 h at RT
4. primary antibody for 1 h at RT; secondary antibody for 1 h at RT
5. primary antibody for 1.25 h at RT and 5 – 8 h at 4 ºC; secondary antibody incubations for 1.5 h at RT.
6. primary antibody for 1.25 h at RT and 5 – 8 h at 4 ºC; secondary antibody incubations for 1.25 h at RT and 5-8 hr at 4 ºC or 1.5 h at RT.

We found that primary antibody incubations times of 1.25 h at room temperature and 5-8 h at 4 ºC, followed by secondary antibody incubation times of either 1.5 hours at room temperature or 1.25 h at room temperature followed by 5 to 8 h at 4 ºC produced nearly identical results. Thus, our optimal long incubation time protocol requires primary antibody incubations overnight at 4 ºC and secondary antibody incubations overnight at 4 ºC, while our optimal short incubation time protocol requires primary antibody incubations for 1.25 h at room temperature and 5-8 h at 4 ºC, followed by either 1.5 h at room temperature or 1.25 hours at room temperature and 5-8 hours at 4 ºC. The resulting optimized antibody conditions and lysates amount to load for each antibody of interest are shown in Table II-1.

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Chapter III. Selective Desensitization of Growth Factor Signaling by Cell Adhesion to Fibronectin

1. Abstract

Cell adhesion to the extracellular matrix is required to execute growth factor-mediated cell behaviors, such as proliferation. A major underlying mechanism is that cell adhesion enhances growth factor-mediated intracellular signals, such as extracellular signal-regulated kinase (Erk). However, because growth factors use distinct mechanisms to activate Ras-Erk signaling, it is unclear whether adhesion-mediated enhancement of Erk signaling is universal to all growth factors. We examined this issue by quantifying the dynamics of Erk signaling induced by epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) in NIH-3T3 fibroblasts. Adhesion to fibronectin(FN)-coated surfaces enhances Erk signaling elicited by EGF, but not by bFGF or PDGF. Unexpectedly, adhesion is not always a positive influence on growth factor-mediated signaling. At critical sub-saturating doses of PDGF or bFGF, cell adhesion ablates Erk signaling; that is, adhesion desensitizes the cell to growth factor stimulation, rendering the signaling pathway unresponsive to growth factor. Interestingly, the timing of growth factor stimulation proved critical to the desensitization process. Erk activation significantly improved only when pre-exposure to adhesion was completely eliminated; thus, concurrent stimulation by growth factor and adhesion was able to partially rescue adhesion-mediated desensitization of PDGF- and bFGF-mediated Erk and Akt signaling. These findings suggest that adhesion-mediated desensitization occurs with rapid kinetics and targets a regulatory point upstream of Ras and proximal to growth factor receptor activation. Thus, adhesion-dependent Erk signaling is not

universal to all growth factors, but rather is growth factor-specific with quantitative features that depend strongly on the dose and timing of growth factor exposure.

2. Introduction

Cell adhesion plays a key role in regulating cellular behaviors such as gene expression, cell survival, and proliferation. Normal cells deprived of adhesion to the extracellular matrix undergo cell cycle arrest (1,2) and programmed cell death, even when soluble growth and survival cues are present (1,43-45). This adhesion-dependence is often de-regulated during cancer development, allowing transformed cells to acquire growth and survival advantages over their normal counterparts (46-48). Adhesion-independent survival and proliferation play a role not only in the build-up of cell mass during tumor formation, but also in the survival of cancer cells in foreign, secondary sites during metastasis (46,49,50).

Because of the physiological importance of adhesion-mediated cell regulation, significant attention has been given to uncovering the underlying signaling mechanisms. One prominent point of crosstalk between adhesion and growth factors involves the serine/threonine kinase, extracellular signal-regulated kinase (Erk). Several reports have shown that growth factor-mediated Erk signaling is enhanced among cells adhered to extracellular matrix (ECM) proteins (2,4,12,13,15-18,24,29,32). In fact, this adhesion-mediated enhancement of Erk signaling plays a crucial role in cell cycle regulation. In NIH-3T3 fibroblasts, suspended cells trigger only a transient Erk signal; however, when

adhered to FN, growth factor treatment supports both a sustained Erk signal and subsequent progression through the cell cycle (24).

Erk is a major signaling protein that is activated by a wide array of stimuli, including several growth factors such as PDGF, bFGF, and EGF (51-53). It is unclear whether adhesion enhances Erk signaling in response to all of these growth factors, or whether only a subset of growth factors signal in an adhesion-dependent manner. Growth factors use substantially different mechanisms to trigger Erk signaling. Unlike EGF, bFGF binding to the cell surface is mediated by two distinct families of cell surface receptors (52). Following ligand binding, EGF receptors are phosphorylated on key tyrosine residues that recruit signaling proteins. In contrast, bFGF receptors phosphorylate the multidocking protein FSR2, which subsequently serves as a scaffold to trigger downstream signaling pathways. In addition to activation pathways, growth factors differ in negative feedback mechanisms that desensitize signaling (54,55). For example, while stimulation via EGF and PDGF result in serine/threonine phosphorylation of their respective receptors, this receptor phosphorylation results in Erk inhibition only in the cells stimulated by PDGF. Interestingly, Erk activation in EGF-stimulated cells remain unaffected (54). Such differences in growth factor signaling mechanisms raise the hypothesis that growth factors may differ in the extent to which their stimulation of Erk signaling is adhesion-dependent.

To begin to test this hypothesis, we measured the effect of cell adhesion on Erk signaling by three growth factors (EGF, bFGF, and PDGF). In order to measure the level

of adhesion-dependence on both the magnitude and dynamics of the Erk signal, we implemented a quantitative protocol for Western blot imaging and analysis. This quantitative approach revealed that adhesion-dependent Erk signaling is selective to EGF in NIH-3T3 fibroblasts. Furthermore, our data reveal that adhesion is not always a positive influence on growth factor-mediated Erk signaling. At a critical sub-saturating dose of PDGF and bFGF, cell adhesion actually thwarts Erk signaling. Our results show that adhesion desensitizes cells from subsequent growth factor-mediated activation of Erk; that is, adhesion renders the signaling pathway unresponsive to growth factor treatment. Interestingly, reducing the duration of cell adhesion prior to growth factor stimulation proved critical in the desensitization process. Thus, PDGF- and bFGF-mediated Erk signaling significantly improved among adherent cells only when pre-exposure to adhesion was completely eliminated; however, concurrent stimulation by growth factors and adhesion was only able to partially neutralize growth factor-mediated desensitization. Our findings suggest that adhesion-dependence of Erk signaling is not universal to all growth factors, but rather is growth factor-specific with quantitative features that depend strongly on the dose and timing of adhesion and growth factor exposure.

3. Experimental Procedures

3.1 *Cell maintenance*

NIH-3T3 fibroblasts (ATTC) were cultured and maintained in 89% DMEM (Gibco), 10% DCS + Fe (Gibco), 1% PenStrep (Gibco). After approximately 2 d of growth, when cells reached between 70-80% confluence, subconfluent cells were suspended using 0.25% trypsin-EDTA (Gibco) and reseeded onto tissue culture dishes.

3.2 *Protein-coating surfaces*

Fibronectin (FN)-coated surfaces were prepared by incubating 2 mL of 5 μg/mL FN (Sigma) diluted in Dulbecco's phosphate buffered saline (dPBS) (Sigma) in tissue culture dishes overnight at 4 ºC. The dishes were gently rocked during adsorption. The dishes were then blocked with 1 mg/mL heat-inactivated bovine serum albumin (BSA) in dPBS for 1 h at 37 ºC. Poly-HEMA (PH)-coated surfaces were prepared by incubating 5 mL of a solution containing 6 mg/mL PH (Sigma) dissolved in 70% biological grade ethanol (Sigma) in uncovered tissue culture dishes overnight at room temperature.

3.3 *Cell adhesion experiments*

NIH-3T3 cells were suspended using 0.25% trypsin-EDTA, reseeded, and grown until 70-80% confluent. Subconfluent dishes were starved in completely serum-free medium (99% DMEM (Gibco), 1% PenStrep (Gibco), 1 mg/mL BSA (Sigma)) for 20 h to bring adhesion signals back to basal levels. Serum-starved cells were suspended using 0.05% trypsin; trypsin activity was quenched by adding soybean trypsin inhibitor (Sigma)

to a final concentration of 0.5 mg/mL. Cells were re-suspended in serum-free medium to a concentration of 5 x 10^5 cells/mL and were either plated onto FN-coated dishes or PH-coated dishes; PH-coated dishes were rocked to prevent cell aggregation.

After cells acclimated to the surface for varying lengths of time (either 2.5 h, 1 h, or 0 h, as described in the text), cells were stimulated with the indicated amount of either PDGF (Sigma), bFGF (Sigma), or EGF (Peprotech). Cells were lysed in buffer containing 50 mM Tris (pH 7.5), 150 mM sodium chloride, 50 mM β-glycerophosphate (pH 7.3), 10 mM sodium pyrophosphate, 30 mM sodium fluoride, 1% Triton X-100, 1 mM benzamidine, 2 mM EGTA, 100 μM sodium orthovanadate, 1 mM dithiothreitol, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 mM PMSF. Lysates were incubated in lysis buffer for 15 min on ice before centrifugation and collection of the supernatant. Micro-BCA Protein Assay Kit (Pierce) was used to determine total protein concentration.

3.4 *Immunoblotting*

Whole cell lysates were resolved by 10% SDS-polyacrylamide gel electrophoresis and blotted onto a PVDF membrane. Blots were probed using either an antibody against dually phosphorylated Erk (Cell Signaling), S473 phosphorylated Akt (Cell Signaling), Erk2 (Santa Cruz), Sos (Santa Cruz), or caspase 3 (Upstate). In the cases of Sos and caspase 3, a 7% and 15% gel were used to better resolve high- and low-molecular-weight proteins, respectively. Blots were imaged and quantified as described in the Results section.

4. Results

4.1 *EGF, but not PDGF and bFGF, induces adhesion-dependent Erk activation*

Using the quantitative Western blotting protocol described in detail in Chapter 2, we measured the Erk signaling response to growth factor treatment of cells adhered on FN or held in suspension. Our initial experiments used growth factor concentrations well above the dissociation constant (K_d) (Table III-1).

Table III-1: Summary of growth factor properties including the critical concentration at which adhesion-mediated desensitization occurs.

Growth Factor	MW (kDa)	K_d (pM)	Critical concentration, $[GF]_c$ (pM)
EGF	6.20	670 (56)	N/A
PDGF	24.6	100-1000 (57-63)	8.1
bFGF	16.4	30 (64,65)[a]	1.2

[a] At low concentrations, bFGF will bind almost exclusively to high-affinity sites (64), thus, the reported K_d corresponds to bFGF interaction with its high-affinity receptor.

At these saturating growth factor concentrations, EGF-mediated Erk signaling is enhanced by cell adhesion. Cells adhered on FN exhibit approximately 3-fold greater Erk activation than cells held in suspension in response to treatment with EGF for 12 min (Figure III-1). Meanwhile, neither bFGF- nor PDGF-mediated Erk signaling at a single early time-point are adhesion-dependent (Figure III-1A). These results suggest that some growth factors (EGF) signal better via the Erk pathway when in an adhesive setting, while other growth factors (PDGF, bFGF) promote Erk signaling in an adhesion-independent manner.

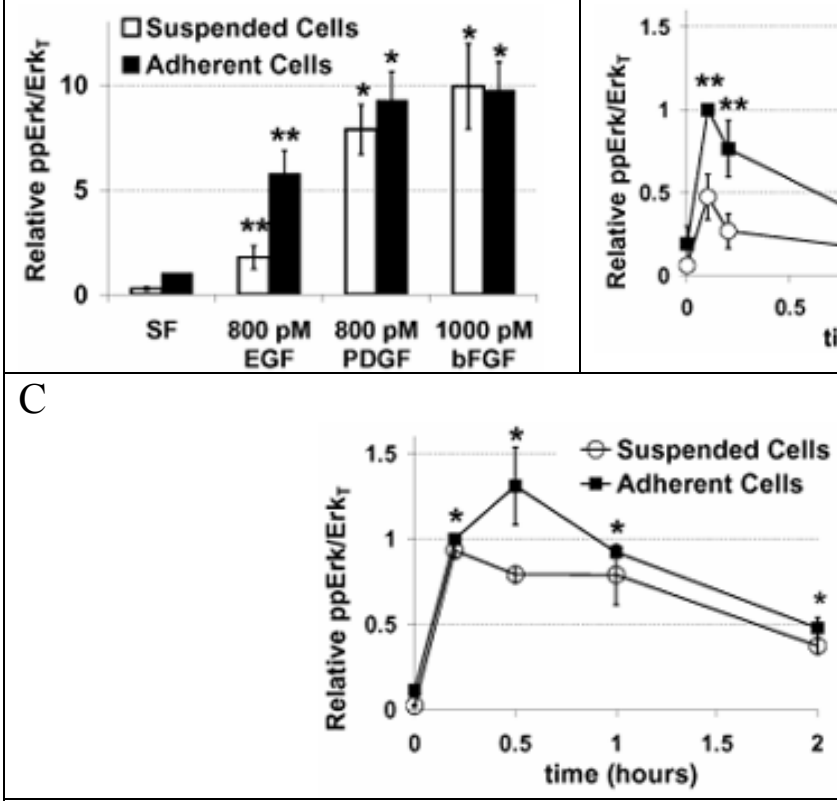

Figure III-1: Adhesion-dependence of Erk signaling at saturating concentrations of growth factors.

Serum-starved cells were either held in suspension via PH-coated dishes or allowed to adhere on FN-coated dishes for 2.5 hr prior to stimulation with serum-free medium containing a single growth factor at the indicated concentrations. After the desired time of exposure to growth factor, cells were lysed, and lysates were analyzed via Western blot with the anti-phospho-Erk and anti-Erk antibodies. The relative amount of active Erk (ppErk) normalized to the equal-loading control, total Erk (Erk_T), is reported for the different treatment conditions. **(A) Adhesion enhances Erk signaling in response to EGF, but not bFGF or PDGF, stimulation.** Cells held in suspension (empty) and those adhered to FN (filled) were stimulated with the indicated growth factor-containing medium or with serum-free medium (SF) and were lysed after 12 min of stimulation. Error bars represent sample standard errors (n = 2-9). The single asterisk denotes that Erk activation in the suspended and adherent cells is statistically similar. The double asterisk denotes that ERK activation in the suspended and adherent cells is statistically different ($P < 0.01$) using Student's t-test. **(B) Adhesion enhances EGF-mediated Erk signaling over the entire time course.** Cells held in suspension (empty circles) or allowed to adhere on FN (solid square) were stimulated with 800 pM EGF for the indicated times. Error bars represent sample standard errors (n=2-4). The double asterisk denotes that ERK activation in the suspended and adherent cells is statistically different with $P < 0.05$ (6 min.) and $P < 0.09$ (12 min.). All P values were computed using Student's t-test. **(C) PDGF activates Erk in an adhesion-independent manner over the entire time course.** Cells held in suspension (empty circles) or allowed to adhere on FN (solid squares) were stimulated with 800 pM PDGF for the indicated times. Error bars represent sample standard errors (n = 2-4). The single asterisk denotes that ERK activation in the suspended and adherent cells is not statistically different using Student's t-test.

These observations are based on a single, early time-point. However, others have shown that growth factor-mediated Erk signaling may exhibit different dynamical features in adhered versus non-adhered cells (15,22,24,29). For example, in both adhered and suspended cells, PDGF stimulates Erk equivalently at early times, but only the adhered cells maintain a sustained Erk signal (22). To examine whether adhesion affects the dynamics of growth factor-mediated Erk signaling, we measured a full time-course of Erk signaling in response to each of the three growth factors. For EGF-stimulated cells, the early phase of Erk activation (< 1 h) is adhesion-dependent, while the late phase of the signal reaches a nearly equivalent, basal signal for both adhered and suspended cells (Figure III-1B). Furthermore, both the adherent and suspended cells reach maximum signal intensity after only 6 min of stimulation. Thus, the kinetics of EGF-induced Erk signaling is similar in both adherent and suspended cells, although signal magnitude is clearly adhesion-dependent.

In contrast, Erk activation in cells stimulated with either PDGF (Figure III-1C) or bFGF (Supplemental Figure VI-1, see page VI-1) was adhesion-independent. For both growth factors, the Erk signal reached a similar maximum after approximately 30 min of stimulation. Furthermore, in the case of PDGF, the Erk signal decays with similar kinetics for both suspended and adhered cells. In the case of bFGF, however, the Erk signal is sustained at near-maximum levels in both adhered and suspended cells. Thus, measurements of the complete dynamics of Erk signaling show that EGF, but not PDGF or bFGF, induces Erk signaling in an adhesion-dependent manner.

4.2 *Sub-saturating doses of PDGF or bFGF reveal negative adhesion-growth factor synergism*

The apparent lack of adhesion-dependence in Erk signaling for bFGF and PDGF may be linked to the fact that high concentrations of the growth factors were used. In this concentration regime, excessive growth factor signaling may overcome the need for cell adhesion. Thus, we hypothesized that for PDGF and bFGF, Erk signaling may be adhesion-dependent if concentrations near or less than K_d (Table III-1) were used. To test this possibility, we measured growth factor-mediated Erk signaling across a broad range of growth factor concentrations.

In the case of EGF, varying its concentration over three orders of magnitude did not affect the observed adhesion-mediated enhancement in Erk signaling (Figure III-2A). Regardless of its concentration, EGF stimulated an approximately 3-fold greater Erk response among adherent cells than among suspended cells (Figure III-2A). In contrast, experiments with different PDGF and bFGF concentrations revealed an unexpected response (Figure III-2B and Figure III-2C). At a critical growth factor concentration (8 pM PDGF or 1 pM bFGF), cells in suspension induced Erk signaling to a significantly greater extent than did adherent cells. Above the critical PDGF and bFGF concentration, adhered and suspended cells responded equivalently (Figure III-2B and Figure III-2C). At the critical PDGF and bFGF concentration, the suspended cells responded 7-fold and 13-fold better, respectively, than their adherent counterparts (Figure III-2B and Figure III-2C). These results reveal a negative synergism between adhesion and growth factor stimulation: adhesion *thwarts* Erk activation at critically low doses of PDGF and bFGF.

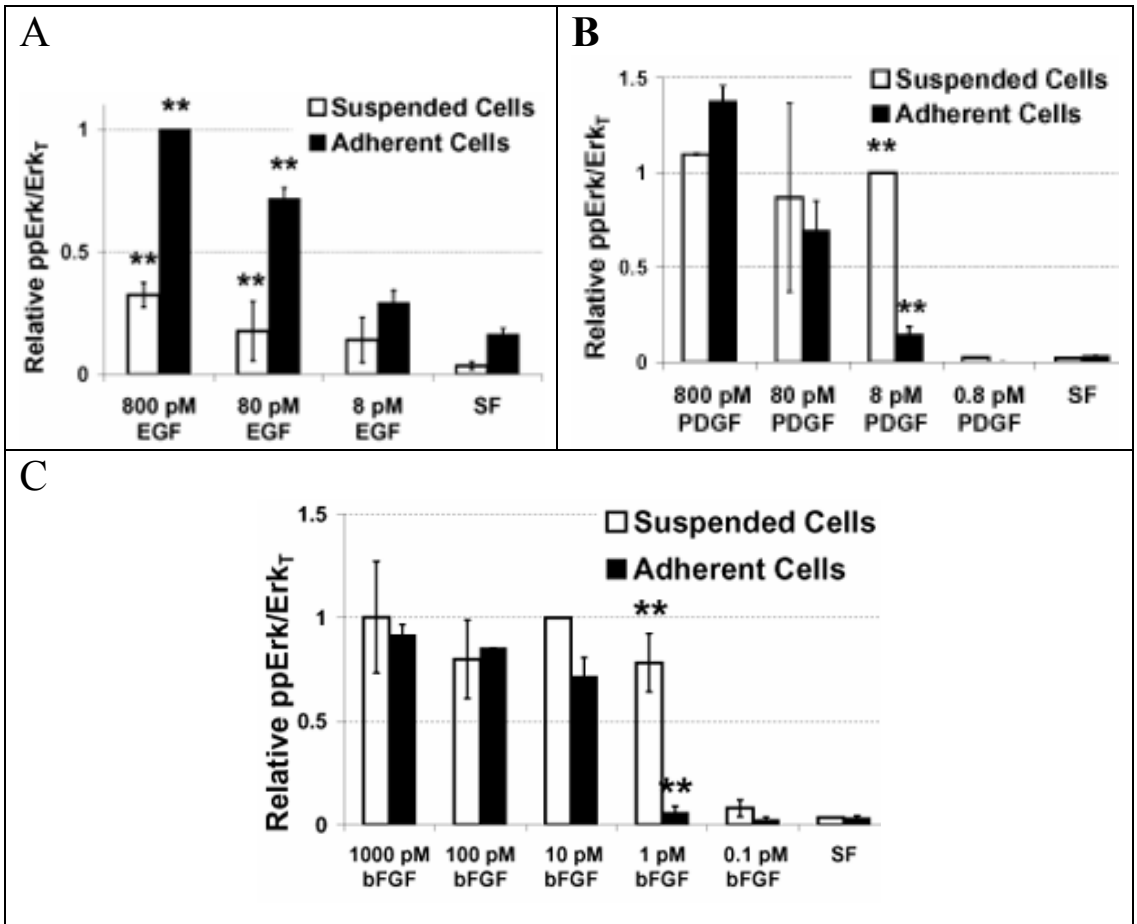

Figure III-2: Adhesion-dependence of Erk signaling across a wide range of growth factor doses.

NIH-3T3 cells were treated as described in the legend to Figure III-1, except that cells were stimulated with serum-free medium containing different doses of (A) EGF for 12 min, (B) PDGF for 30 min or (C) bFGF for 30 min. The response of cells held in suspension (empty) is compared to cells adhered on FN (filled). (A) Error bars represent sample standard errors (n=2-4). The double asterisk denotes that ERK activation in suspended and adherent cells is statistically different with $P < 0.001$ (800 pm) and $P < 0.07$ (80 pm). (B) Error bars represent sample standard errors (n=2-4). The double asterisk denotes that ERK activation in suspended and adherent cells is statistically different with $P < 0.04$. (C) Error bars represent sample standard errors (n=2-4). The double asterisk denotes that ERK activation in the suspended cells is statically different with $P < 0.07$. All P values were computed using Student's t-test.

Since this negative synergy was observed at a specific time point in Erk signaling, we investigated the dynamics of Erk signaling more completely at the critical PDGF

concentration (Figure III-3) and bFGF concentrations (Supplemental Figure VI-2, see page VI-1). At the critical PDGF concentration, adherent cells were unable to induce Erk signaling during the entire time course (Figure III-3). In contrast, Erk signaling in suspended cells was substantial throughout the entire time course (Figure III-3). Thus, the observed negative synergism between adhesion and growth factor stimulation is not an artifact of selecting a specific time point; rather, the entire dynamics of PDGF-mediated Erk signaling is suppressed among adherent cells at the critical PDGF concentration. Similar results were observed for bFGF as adhesion completely ablated bFGF-mediated Erk activation (Supplemental Figure VI-2, see page VI-1).

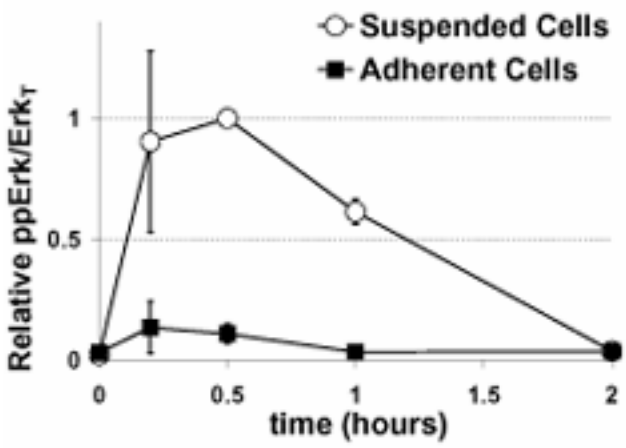

Figure III-3: Time course of adhesion-dependent Erk signaling at the critical PDGF concentration.

Serum-starved NIH-3T3 cells were held in suspension (empty circles) or adhered on FN (solid squares) as described in the legend to Figure III-1. Cells were stimulated with serum-free medium containing 8 pM PDGF and lysed at the indicated times. The relative amount of active Erk normalized to total Erk is reported. Error bars represent sample standard errors (n = 2-4).

4.3 *Adhesion desensitizes PDGF- and bFGF-mediated Erk signaling*

The observed negative synergy reveals that cell adhesion to FN selectively abrogates PDGF- and bFGF-mediated Erk signaling when these growth factors are

present at low concentrations. These observations raise the possibility that adhesion desensitizes subsequent Erk activation. Desensitization refers to a state in which a signaling pathway becomes unresponsive to stimuli. This desensitized or refractory state may occur when a signal triggers negative feedback mechanisms that persist and prevent re-activation of the signal in response to new stimuli. Published reports have shown that growth factor stimulation inhibits Erk signaling in response to a second challenge of growth factor (66-68). Our results suggest that adhesion to FN may also desensitize Erk signaling to a select subset of growth factors (PDGF and bFGF).

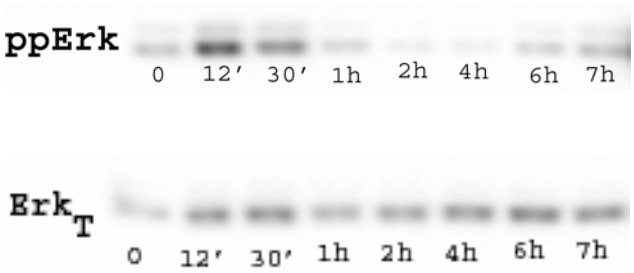

Figure III-4: Adhesion-mediated Erk activation.

Serum-starved NIH-3T3 cells were suspended and re-plated on FN-coated plates as described in the legend of Figure III-1. Cells were lysed at the indicated times after plating without growth factor stimulation. Lysates were analyzed by SDS-PAGE and Western blotting with an anti-phospho-Erk antibody (top panel) and an anti-Erk antibody (bottom panel) as an equal loading control.

Consistent with this hypothesis of adhesion-mediated desensitization, cell adhesion to FN in the absence of growth factors promotes Erk activation (Figure III-4). Adhesion rapidly stimulates the Erk pathway with maximal activation occurring by approximately 12 min after cell seeding. This adhesion-mediated Erk signaling may trigger negative feedback loops that desensitize cells to subsequent Erk signaling by

PDGF and bFGF. If desensitization is responsible for reduced PDGF- and bFGF-mediated Erk signaling, then reducing the duration of cell adhesion prior to growth factor stimulation might alleviate this suppression. As cells were seeded 2.5 hr prior to

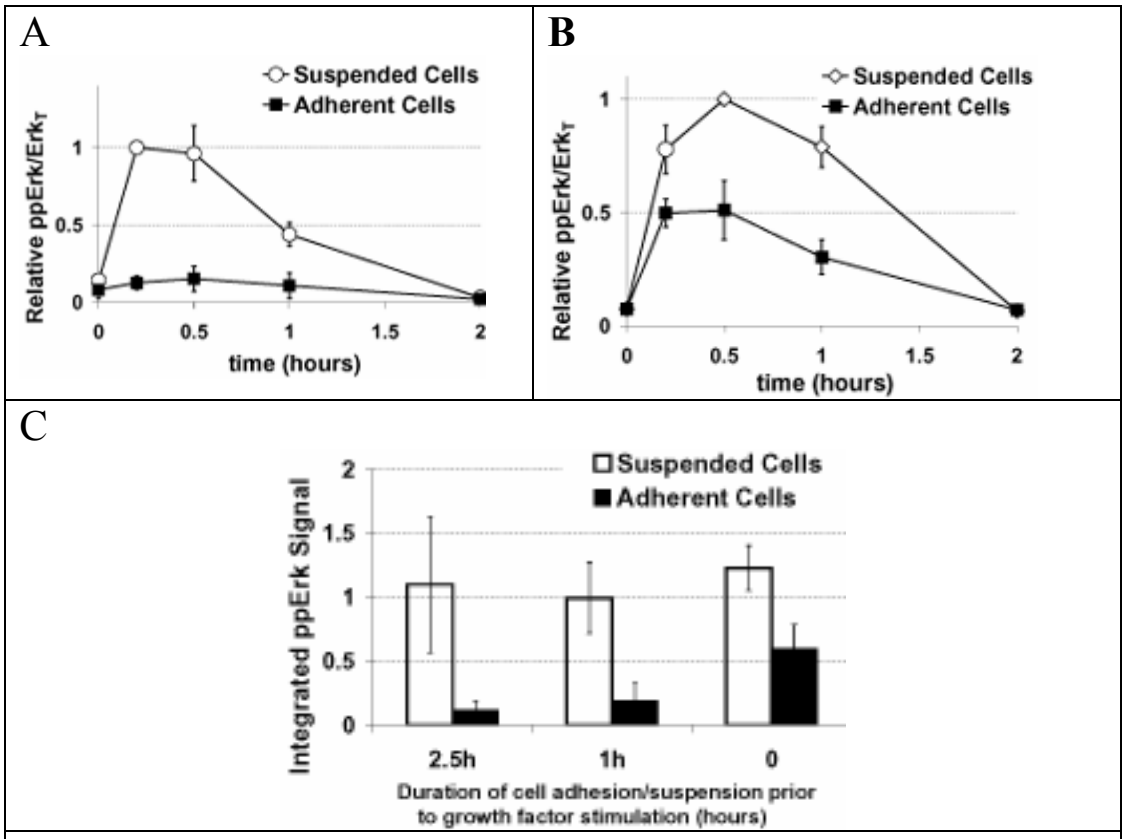

Figure III-5: The dependence of PDGF-mediated ERK signaling on the duration of pre-exposure to FN-coated surfaces.

Serum-starved NIH-3T3 cells were held in suspension (empty circles) or adhered on FN-coated plates (solid squares) as described in the legend to Figure III-1. The duration cells spent in suspension or adhered to FN prior to stimulation with 8 pM PDGF was reduced from 2.5 h to (A) 1 h or (B) 0 h. The integral of the ERK time-course for all three acclimation times are shown in (C). For A and B, error bars represent sample standard errors (n = 3-6). For C, the error bars represent propagated error when the trapezoid rule is used to calculate the integrated signal.

stimulation in all previous experiments, we tested this hypothesis by measuring PDGF-mediated Erk signaling among cells that were exposed to FN-coated surfaces for shorter

times, specifically 1 h and 0 h (Figure III-5A and Figure III-5B, respectively). In the 0 h case, cells were concurrently stimulated with growth factor and plated onto FN-coated dishes.

Reducing the duration of adhesion significantly enhanced PDGF-mediated Erk signaling among adherent cells. While Erk signaling was severely attenuated among cells that had adhered for 2.5 h (Figure III-3), reducing adhesion time to 1 h only slightly improved Erk signaling (Figure III-5A). However, eliminating pre-exposure to adhesion altogether by concurrent stimulation with PDGF significantly improved Erk signaling among adherent cells (Figure III-5B). In order to quantify the enhancement in PDGF-mediated Erk signaling in response to decreasing the duration of adhesion, we integrated the time course of Erk signaling for cells held in suspension or adhered on FN for 0 h, 1 h and 2.5 h (Figure III-5C). When the pre-exposure time to adhesion is reduced from 2.5 h to 0 h, the integrated Erk signal increases approximately 5-fold. Notably, even concurrent stimulation was unable to rescue PDGF-mediated Erk activation to the same level as that observed in suspended cells, suggesting that adhesion-mediated desensitization occurs rapidly. The rapid timescale of adhesion-mediated desensitization is consistent with the fact that cell adhesion to FN significantly activates Erk within 12 min of cell seeding (Figure III-4).

4.4 *Mechanisms underlying adhesion-mediated desensitization*

The hyperphosphorylation of Sos is a prominent mechanism in growth factor-mediated desensitization of Erk (69-73). To determine whether cell adhesion to FN

desensitizes the Erk pathway in a similar manner, we measured the effect of cell adhesion on the hyperphosphorylation of Sos using a gel retardation assay (Figure III-6). The mobility of Sos did not change among cells that were plated on FN-coated substrates in the absence of growth factor (Figure III-6, lanes 1-7). In contrast, a positive-control treatment with PDGF induces a clear and significant retardation in Sos mobility (Figure III-6, lane 10). Thus, adhesion-mediated desensitization of Erk signaling does not involve hyperphosphorylation of Sos.

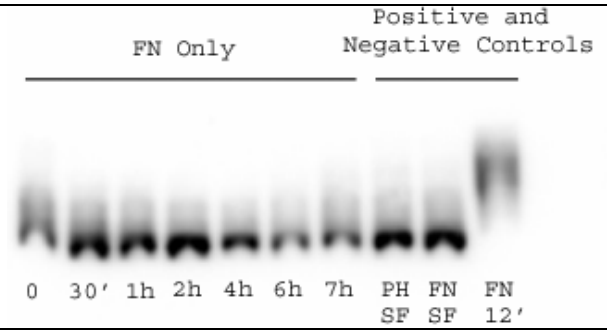

Figure III-6: The effect of cell adhesion on Sos hyperphosphorylation.

Serum-starved NIH-3T3 cells were suspended and re-plated on FN-coated plates as described in the legend of Figure III-1. Cells were lysed at the indicated times after plating without growth factor stimulation. Lysates were analyzed by SDS-PAGE and Western blotting with an anti-Sos antibody. A shift in total Sos indicates presence of the hyperphosphorylated form of Sos. Lanes 8 and 9 represent negative unstimulated controls for Sos hyperphosphorylation. Lane 8 contains cells that have been held in suspension for 2.5 h, while lane 9 represents cells that have been adhered to FN to 2.5 h. Lane 10 represents the positive control for the hyperphosphorylated form of Sos and contains cells that have been adhered to fibronectin for 2.5 h prior to stimulation by 800 pM PDGF for 12 min.

To determine whether adhesion-mediated suppression of PDGF and bFGF signaling was specific to the Erk pathway, we measured Akt signaling under similar conditions. PDGF- and bFGF-mediated Akt phosphorylation was also significantly diminished among adherent cells (Figure III-7 and Supplemental Figure VI-3 on page VI-2, respectively). In addition, PDGF-mediated Akt activation among adherent cells significantly improved as adhesion time on FN was decreased (Figure III-7). While

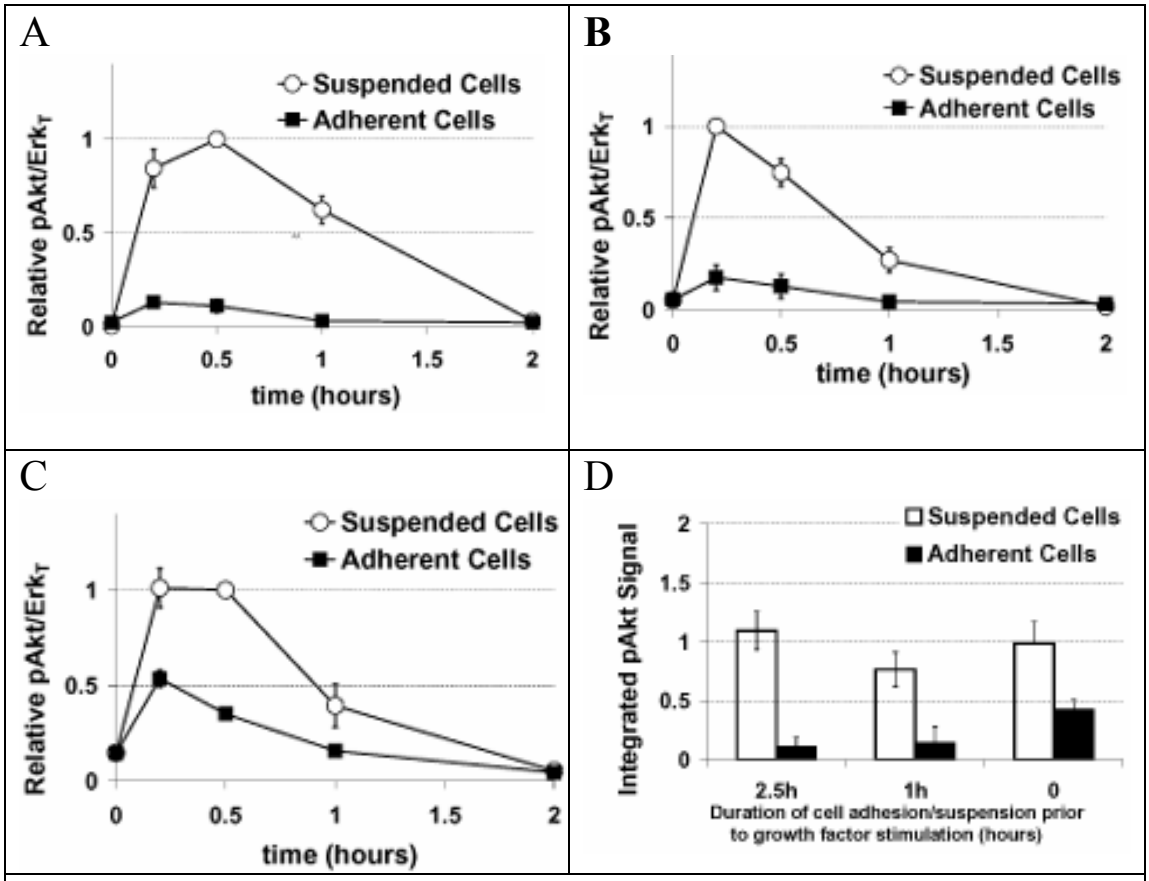

Figure III-7: Adhesion-dependence of PDGF-mediated Akt signaling.

Serum-starved NIH-3T3 cells were held in suspension (empty circles) or adhered on FN (solid squares) for 2.5 h as described in the legend to Figure III-1. Cells were then treated with PDGF and lysed at the indicated time-points. The level of phosphorylated Akt (pAkt) was quantified and normalized to the amount of total cellular Erk (Erk_T). The duration for which cells were either held in suspension or adhered on FN was reduced from (A) 2.5 h to (B) 1 h or (C) 0 h. The integrated area for all three acclimation times is shown in (D). For A and B, error bars represent the sample standard errors (n = 3-6). For D, the error bars represent propagated standard error when the trapezoid rule is used to approximate the integrated signal.

reducing the duration of adhesion from 2.5 h to 1 h only slightly improved Akt signaling (compare Figure III-7A and Figure III-7B), concurrent stimulation significantly improved Akt signaling among adherent cells (Figure III-7C). Indeed, the integrated Akt signal shows a trend identical to that of the integrated Erk signal. While the integrated Akt

signal for suspended cells remains constant, for adherent cells, the signal clearly increases as the duration of adhesion on FN is reduced (Figure III-7D). Because adhesion suppresses both Erk and Akt signaling, it suggests that adhesion-mediated desensitization of PDGF and bFGF signaling may occur at or above the level of Ras activation, but independent of Sos regulation.

5. Discussion

This study demonstrates that cell adhesion has quantitatively intricate effects on growth factor-mediated Erk signaling. We report that the effect of cell adhesion is specific to the type of growth factor, its dose, and the timing of stimulation. Our system exclusively uses NIH-3T3 fibroblasts that are stimulated in defined medium. We find that adhesion to FN selectively enhances Erk signaling elicited by EGF, but has no effect on bFGF- or PDGF-mediated Erk activation. Unexpectedly at concentrations of PDGF and bFGF (GF_c) that are significantly less than K_d (Table III-1), cell adhesion severely attenuates growth factor-mediated Erk signaling. Thus, adhesion not only enhances cell response to specific growth factors, but also filters out potentially noisy signals from low levels of growth factor. This aspect of adhesion-growth factor crosstalk may play an important role in buffering cell response to noisy background levels of growth factor stimulation. These results reveal that the crosstalk between adhesion and growth factor signaling has intricate quantitative features, consistent with the extensive connectivity between adhesion and growth factor signaling pathways (53,74,75).

Our observation that adhesion to FN enhances EGF-mediated Erk signaling is consistent with other reports (21). Our results further demonstrate that adhesion does not enhance either PDGF- or bFGF-mediated Erk signaling in NIH-3T3 cells, a finding that is contrary to some reports (15,22,24,29). In one such report, bFGF treatment was found to induce sustained Erk signaling that supports cell cycle progression of NIH-3T3 fibroblasts seeded on FN-coated surfaces (76). However, the 3T3 cells used express exogenous human $\alpha_5\beta_1$ integrin, whereas our cell system expresses only endogenous integrin adhesion receptors. Furthermore, both the aforementioned study and others using NIH-3T3 cells supplement the growth factor-containing medium with serum (24,29). This serum supplement is essential to maintain long-term cell viability, a clear requirement for studying cell cycle progression. Our studies, in contrast, employ serum-free medium supplemented with specific growth factors. We have carefully assayed cell death under serum-free conditions by trypan blue staining and by Western blotting for caspase 3 cleavage (data not shown). Our measurements show that cells held in suspension or adhered on FN-coated plates remain viable for 4-5 hr in serum-free conditions. Thus, all reported results are gathered in this time window and offer a clear indication of how Erk signaling by each growth factor is influenced by adhesion without confounding contributions from serum.

In addition to serum, cell type differences may also contribute to apparent differences in adhesion-dependence of Erk signaling. Kazlauskas and colleagues showed that PDGF treatment of mouse embryo fibroblasts (MEFs) adhered on FN induces sustained Erk activation, whereas cells seeded on poly-L-lysine support only a transient

Erk signal (22). The difference between our results and those of Kazlauskas and colleagues may be due to the use of MEF versus NIH-3T3 cells. Moreover, the MEF strain used in the study lacks PDGFRα and expresses endogenous PDGFRβ; our NIH-3T3 cells express both isoforms. Thus, the complement of homo- and heterodimer receptors available for binding PDGF-BB and for intracellular signaling are likely to be different in these two cell types.

Because of both the differences in cell types and receptor expression profiles, as well as the potentially confounding contributions from serum, it remains unclear whether adhesion-dependent Erk signaling is specific to particular growth factors. We sought to address this issue by developing a system that utilizes only NIH-3T3 fibroblast cells expressing endogenous integrins. Furthermore, growth factor stimulation was limited to use of only one growth factor in defined medium, thus no serum was used during the course of the experiments. Thus, our data begins to provide a systematic comparison of the crosstalk between adhesion and three different growth factors. We show that adhesion to FN enhances Erk signaling elicited by EGF, but not by bFGF and PDGF. Our observation that adhesion-mediated enhancement of Erk signaling is not a universal property of all growth factors is consistent with our previous findings in Chinese hamster ovary (CHO) cells. In these cells, the dynamics and magnitude of insulin-mediated Erk signaling are unaffected by cell adhesion to FN (14). Rather, adhesion and insulin synergistically affected IRS-1 phosphorylation en route to co-regulating cell cycle activity.

The specificity of adhesion-dependence might be explained by intrinsic differences in how the receptors of these growth factors signal to Erk. Although all three growth factors employ the canonical Ras-MAP kinase cascade to activate Erk, there are significant differences in the upstream machinery that connect to the core Ras/MAP kinase signaling module. While EGF receptors recruit the necessary signaling proteins mostly by themselves (52), FGF receptors rely on the formation of a multidocking signaling protein complex to recruit the majority of signaling components (77). In addition, bFGF binds to two distinct families of cell surface receptors, the first being the bFGF receptor tyrosine kinase and the second being heparin sulfate proteoglycans (HSPG) (65,78). The binding to and signaling from two distinct receptor families provides additional layers of control and complexity to bFGF-mediated signaling to Erk (79).

In addition to utilizing different mechanisms for activating the Ras/MAPK module, growth factor receptors differ in their susceptibility to negative regulatory mechanisms. For example, serine/threonine phosphorylation of EGF and PDGF receptors has been shown to affect the two receptors differently. G protein-coupled receptor kinase 2 (GRK2) mediated serine/threonine phosphorylation of the PDGF receptor results in a decrease in PDGF receptor tyrosine phosphorylation, which correlates to an observed decrease in Erk activation by PDGF stimulation (54). In contrast, GRK2 mediated serine/threonine phosphorylation has no effect on the tyrosine phosphorylation of the EGF receptor and subsequent Erk activation is also not affected. In summary, there are distinct pathways by which growth factors activate the Ras/MAPK

module as well as differences in growth factor receptor sensitivity to negative regulatory mechanisms. Cell adhesion may also couple to pathways unique to EGF, thereby selectively enhancing EGF-mediated Erk signaling.

While adhesion selectively enhances EGF-mediated Erk signaling at saturating growth factor concentrations, an intriguing feature of adhesion-dependence was found at low, sub-saturating doses of growth factors. PDGF- and bFGF-mediated Erk signaling is substantially attenuated among cells adhered on FN. This adhesion-mediated suppression of growth factor-induced Erk signaling is alleviated if the duration of cell adhesion is reduced. These observations suggest that cell adhesion rapidly triggers mechanisms that desensitize Erk signaling by low concentrations of PDGF and bFGF.

Desensitization of the ERK signaling pathway has been reported in response to growth factor stimulation. Growth hormone (GH) induces Erk activation in HA cells; however, re-exposure to GH in cells that have been pre-treated with this growth factor for 3 h fails to stimulate ERK (67). Similar desensitization of Erk activation has been shown in insulin-treated CHO/IR cells. Although Erk activation occurs upon initial insulin exposure, a second exposure to insulin fails to induce Erk signaling (68). Comparable insulin-mediated desensitization has been observed in 3T3-LI adipocyte cells (66). However, in these cells, insulin pre-treatment does not desensitize EGF-mediated ERK activation. In contrast to insulin and EGF, heterologous desensitization has been observed between EGF and PDGF (80). Swiss-3T3 cells first exposed to PDGF fail to induce Erk activation upon a subsequent treatment with either PDGF or EGF. The

converse is also observed: initial exposure to EGF inhibits subsequent stimulation of ERK signaling by either PDGF or EGF treatment. Hence, there is precedent for desensitization to selectively affect a subset of growth factors.

A prominent mechanism by which growth factor desensitizes ERK signaling in response to subsequent growth factor stimulation involves Sos hyperphosphorylation (69-72). However, our results demonstrate that adhesion to FN does not induce Sos hyperphosphorylation, suggesting that adhesion-mediated desensitization does not occur at the level of Sos regulation.

Our results suggest that the time scale of desensitization is remarkably rapid. Thus, although Sos is not the target of desensitization, another signal extremely proximal to growth factor detection must be involved. Consistent with this possibility, our results show that both Akt and Erk signaling are subject to adhesion-mediated desensitization, suggesting that Ras or some other common upstream element is the point of desensitization. In fact, several growth factor receptors directly interact with adhesion receptors (81). While the association of growth factor receptors with adhesion receptors has been predominantly correlated with positive synergism, it may also sequester and inhibit the activity of low levels of ligand-bound growth factor receptors. Indeed, such heterologous desensitization by receptor sequestration has been demonstrated for EGFR and PDGFR (80). Another possible mechanism of growth factor desensitization may involve direct interactions between growth factors and ECM proteins. Sequestration of TGFβ, VEGF, and HGF by ECM is well documented (82). In fact, VEGF has recently

been shown to bind specific sites on FN, suggesting potential sequestration in more basic reconstituted systems. Thus, ECM protein-mediated sequestration may play a role in diminishing growth factor-mediated signaling on FN-coated dishes, especially in systems employing low doses of growth factor (83).

While elucidating the precise role of these mechanisms is the subject of ongoing work in our lab, it is especially intriguing that adhesion-mediated desensitization occurs selectively at low growth factor concentrations. Thus, adhesion may play an important role in buffering cell response to noisy, background levels of growth factor stimulation. Combined with the ability to enhance signaling for select growth factors, adhesion may have a net positive effect on the signal- to-noise ratio of detecting and responding to growth factors. Deciphering these and other quantitatively intricate ways in which cell adhesion influences growth factor signaling will be crucial to developing a better understanding of how the adhesive microenvironment 'primes' cell behaviors. Such quantitative insight will be important in designing synthetic microenvironments for applications such as tissue engineering and regenerative medicine. In addition, quantification will enable improved assessment of the quality and effectiveness of both biomaterials and cancer therapies.

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Chapter IV. Modeling-Guided Inference of Mechanisms Underlying Erk Signaling Dynamics

1. Abstract

We developed a simple coarse-grain mathematical model to elucidate how specific molecular mechanisms contribute to the dynamics of Erk signaling. Model results are compared to experimental Erk signaling profiles to infer potential mechanisms and to direct future experiments. Our model lumps Erk activation and deactivation mechanisms into four distinct biological motifs: activation, constitutive direct deactivation, feedback-mediated direct-deactivation (a feedback mechanism where the active signal upregulates a protein that deactivates it), and feedback-decoupling deactivation (a feedback mechanism where the active signal deactivates an upstream protein necessary for signal activation). In the presence of activation stimulus, two types of kinetic profiles are observed: (1) transient, where the steady-state signal returns to a basal level, and (2) sustained, where the steady-state signal is maintained to at least its half-maximal level. Our model reveals that feedback-decoupling deactivation and one form of direct-deactivation are necessary to generate a transient signal, while feedback-decoupling deactivation in isolation or any form(s) of direct-deactivation results in sustained signal activation. Notably, our model predicts that signal magnitude enhancement/depression is most significantly impacted by differences in the activation rate constant (κ_{act}). As a difference in peak signal magnitude was experimentally observed for adhesion-dependent Erk activation by EGF-stimulation, our model predicts that adhesion-induced enhancement is the most likely explanation. In addition, our

model provides insight into potential mechanisms responsible for the difference in Erk activation dynamics in adherent cells stimulated by distinct growth factors. In order to determine the mechanistic details responsible for the PDGF/EGF-induced transient Erk activation verses the bFGF-induced sustained Erk activation observed for adherent cells, a difference in the deactivation motifs acting on these adherent cells must be determined. We find that the mechanism responsible for the distinct signaling dynamics resulting from stimulation by different growth factors in adherent cells must be due to either feedback-decoupling deactivation (for the case where no feedback-decoupling deactivation occurs in cells stimulated via bFGF) or direct-deactivation (for the case where feedback-decoupling deactivation is the only form of deactivation present in cells stimulated via bFGF).

2. Introduction

The combination of adhesion to the extracellular matrix (ECM) and growth factors plays a key role in regulating important cellular functions such as proliferation, cell survival, and gene expression. Because Erk activation has been established as a prominent point of cross-talk between adhesion and growth factors, extensive effort has been invested in understanding adhesion-mediated Erk signaling. Synergy in adhesion- and growth factor-mediated Erk activation has been described as equivalent (Galownia, 2006; Mettouchi et al., 2001), as a difference in magnitudes (Asthagiri et al., 2000; Marshall, 1995), as a difference in dynamics from transient to sustained (DeMali et al., 1999; Roovers et al., 1999; Tombes et al., 1998), or not been given any kinetic description at all (Lin et al., 1997; Renshaw et al., 1997; Schwartz and Assoian, 2001). For clarity, several phrases used to describe signal kinetics will be given precise

definitions. In order for a signal to be classified as 'transient,' the temporal profile must return to basal. Alternatively, to be classified as 'sustained,' the temporal profile must reach and maintain a signal equal to at least half-maximum. The phrases 'maximum intensity' and 'peak intensity' will be used to describe the largest magnitude in the temporal profile.

Because both the kinetic profile and signal magnitude of Erk activation may be affected, synergy in Erk signaling likely involves crosstalk not only in activation mechanisms, but also across Erk deactivation pathways (Asthagiri and Lauffenburger, 2001). Dephosphorylation due to basal phosphatase activity regulates Erk deactivation (Keyse, 2000; Lewis et al., 1998; Tamura et al., 2002), a mechanism we term as constitutive direct deactivation. In addition, activation of Erk itself catalyzes its deactivation via two separate feedback mechanisms where active Erk: (1) upregulates phosphatase levels (Brondello et al., 1997; Grumont et al., 1996) that deactivate it, a feedback mechanism we term feedback-mediated direct deactivation, or (2) deactivates its upstream activators (Brunet et al., 1994; Buday et al., 1995; Cherniack et al., 1995; Dong et al., 1996; Langlois et al., 1995; Wartmann et al., 1997), a feedback mechanism we term feedback-decoupling deactivation.

Our experimental data reveals that adhesion-dependence of Erk signaling is not universal to all growth factors, but rather is growth factor-specific (Galownia, 2006). In addition, our experimental data indicates that different growth factors result in distinct kinetic Erk activation profiles. However, precisely what combination of crosstalk

between integrins and growth factors underlies the observed synergy in Erk signaling remains unclear. Therefore, in this study we developed a computational model to investigate how different activation and deactivation motifs within the Erk signaling network contribute to Erk signaling dynamics. Our model predictions are then compared to experimental data to narrow down potential mechanism(s) producing the observed kinetic profiles.

3. Background

3.1 *Activation of MAPK pathway*

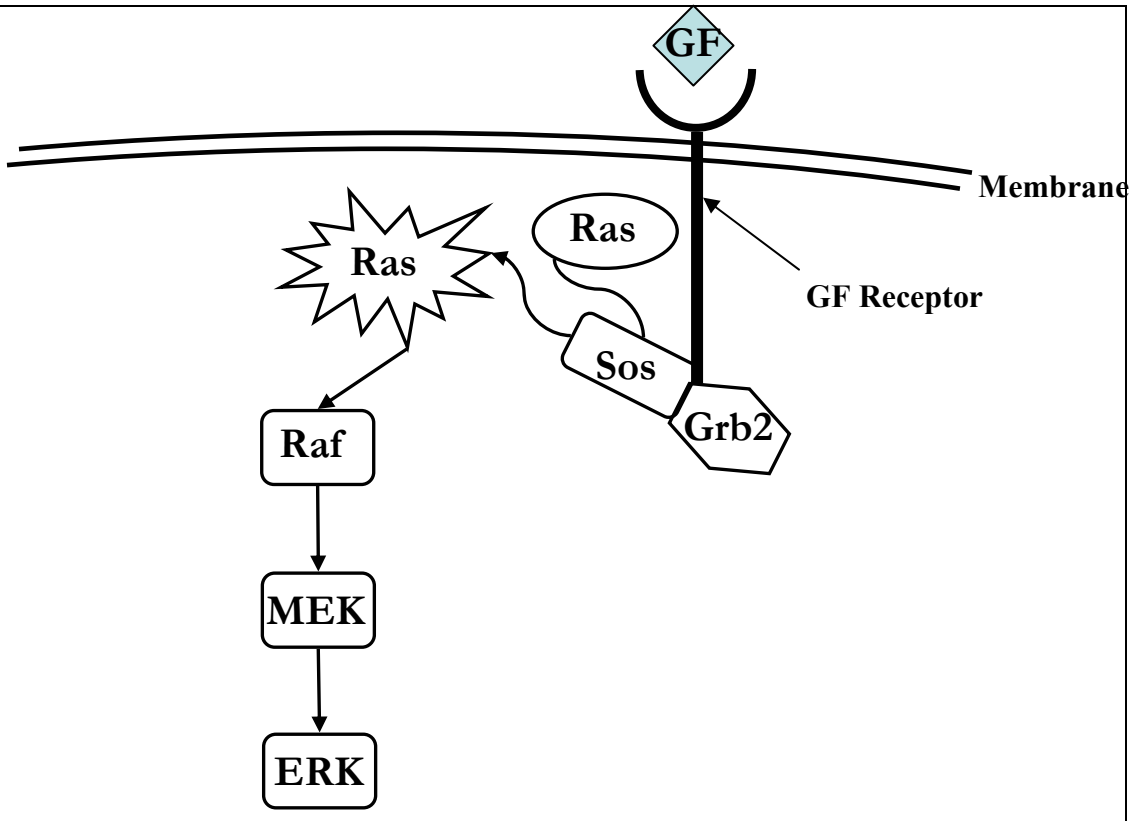

Figure IV-1: Canonical MAPK pathway
Growth Factor-receptor binding stimulates receptor autophosphorylation, which enables Grb2 binding. Grb2 then recruits Sos, allowing Sos-mediated Ras activation. Activated Ras triggers a signaling cascade by activating Raf. Raf phosphorylates Mek, which phosphorylates Erk.

In the canonical growth factor-MAPK (mitogen activated protein kinase) pathway (Figure IV-1), growth factor ligands bind to receptors and elicit tyrosine autophosphorylation of the receptor (Ford and Pardee, 1999; Roovers and Assoian, 2000). This enables growth factor receptor bound protein 2 (Grb2) to bind to the receptor and recruit the guanosine 5'-triphosphate exchange factor, son-of-sevenless (Sos) (Roovers and Assoian, 2000). Sos-mediated nucleotide exchange activates the Ras G-protein by converting it to its GTP form, which enables it to bind to and activate Raf kinase. Raf kinase then activates Mek kinase, which activates Erk kinase and eventually leads to S-phase entry (Ford and Pardee, 1999; Roovers and Assoian, 2000).

One pathway by which integrins enhance MAPK activation is through direct interaction with growth factor receptors themselves (Giancotti and Tarone, 2003; Miyamoto et al., 1996). Integrin-mediated cell adhesion has been shown to induce activation of insulin receptor, EGF receptor, PDGF receptor, and bFGF receptor (Giancotti and Tarone, 2003; Schlessinger, 2000). Recent studies have uncovered multiple mechanisms by which integrins induce MAPK activation at the level of receptor protein tyrosine kinase activation (Giancotti and Tarone, 2003). In one such mechanism, integrin-mediated adhesion has been shown to induce growth factor receptor aggregation and the subsequent activation of the MAPK pathway (Miyamoto et al., 1996).

Another mechanism involves integrin-mediated activation of Src. In this form of regulation, a macromolecular complex comprised of the cytoplasmic tail of the EGF receptor, $p130^{Cas}$, and Src form in response to cell adhesion. Src is required for complex

assembly, and complex formation is necessary to enable Src-mediated phosphorylation and activation of the EGF receptor. Once in complex, Src phosphorylates the cytoplasmic domain of the receptor on tyrosine residues 845, 108, and 1173, resulting in its activation. Interestingly, Src is unable to phosphorylate the receptor on Tyr^{1148}, which is a major site of the receptor phosphorylation by EGF, indicating that distinct mechanisms exist by which integrins and growth factors activate the growth factor receptor (Moro et al., 2002).

Alternatively, for integrin- and Src-family kinase-mediated PDGF receptor activation, active SFK phosphorylates SIRP-α/SHPS-1, leading to the recruitment of the tyrosine phosphatase, SHP-2, to the cell's membrane. SHP-2 then associates with PDGF receptor and dephosphorylates it at the tyrosine residue mediating binding to Ras-Gap, a known negative regulator of Ras. Ras-Gap activation is efficiently reduced, leading to both Ras and subsequent Erk activation.

There are also several mechanisms by which integrin-mediated activation of focal adhesion kinase (Fak) activates the MAPK pathway. Although Fak activation is not well understood (Giancotti and Tarone, 2003; Lee and Juliano, 2004), it is known that Fak is recruited to focal adhesion complexes and is activated via autophosphorylation on Tyr^{397}, thus creating a binding site for the Src homology 2 (SH2) domain of the Src-family kinases, Src or Fyn (Giancotti and Ruoslahti, 1999; Giancotti and Tarone, 2003; Lee and Juliano, 2004; Schlaepfer et al., 1994).

In the first integrin-mediated Fak kinase activation mechanism, Cas and paxillin are also recruited to the membrane via integrin-mediated cell adhesion (Hanks and Polte, 1997). The combined kinase activity of Fak and the Src-family kinases results in the phosphorylation of multiple sites on Fak, Cas, and paxillin (Hanks and Polte, 1997; Vuori, 1998). Phosphorylation of Fak by Src at tyrosine 925 (Tyr^{925}) (Giancotti and Ruoslahti, 1999; Lee and Juliano, 2004; Schlaepfer et al., 1994) creates a binding site for the Grb2-Sos complex, thereby providing the link between integrin-mediated signaling and the canonical MAPK pathway (Giancotti and Ruoslahti, 1999; Lee and Juliano, 2004; Renshaw et al., 1999; Schlaepfer et al., 1994; Schwartz and Assoian, 2001).

In the second integrin-mediated Fak kinase activation pathway, $p130^{Cas}$ interacts with Fak through its SH3 domain and is phosphorylated by Src. This enables recruitment of Crk. Crk associates with either Sos or the guaneonucleotide exchange factor C3G for Rap-1, resulting in the activation of B-Raf. Notably, B-Raf has been linked to Erk activation (Barberis et al., 2000), thus providing another pathway linking Fak to MAPK activation in cells that express B-Raf (Giancotti and Tarone, 2003; Lee and Juliano, 2004).

Another integrin-mediated mechanism that affects MAPK signaling occurs via Fak independent activation of the p21-activated kinase (Pak) (Howe et al., 2002). Integrin-mediated adhesion relieves the inhibition of protein kinase A (PKA) on Pak (Giancotti and Tarone, 2003). In addition, focal adhesion complexes recruit active Rac, which then associates with and activates Pak (Giancotti and Tarone, 2003; Howe et al.,

2002). Once active, Pak3 phosphorylates Raf1, while Pak1 phosphorylates Mek1 (Slack-Davis et al., 2003). Thus a link between Pak and activation of the MAPK pathway is established.

A fifth mechanism by which integrins affect MAPK signaling involves Src-family kinases, such as Fyn or Yes (Giancotti and Ruoslahti, 1999; Giancotti and Tarone, 2003). Specific α integrin subunits, including α_1, α_5, α_v, bind to the transmembrane adapter protein caveolin-1 independently of Fak, through their external and transmembrane domains (Giancotti and Tarone, 2003; Lee and Juliano, 2004). Caveolin-1 links the transmembrane portion of the integrin α subunit to the Src-family kinase. While the mechanism by which integrin engagement induces the Src-family kinase activation is not well understood, it is known that the activated Src-family kinase undergoes a conformational change to expose its Src homology 3 (SH3)-binding domain. The SH3-binding domain recruits Shc, which is then phosphorylated at Tyr^{317}. The Grb2-Sos complex then binds to Shc, and ultimately induces Erk activation (Giancotti and Tarone, 2003; Lee and Juliano, 2004), providing yet another link to MAPK activation.

A sixth pathway by which integrins activate the MAPK pathway involves the Src family tyrosine kinase Syk (Miranti et al., 1998). Prior to activation of Syk, the β_3 integrin subunits are associated with Csk, enabling Csk to phosphorylate the C-terminal autoinhibitory site of Src-family kinases; thus all integrin-associated Src-family kinases have been rendered inactive (Giancotti and Tarone, 2003). Upon ECM binding, Csk is released from the β_3 subunit, enabling activation of integrin-associated Src-family kinases

and subsequent recruitment and activation of Syk and ZAP70 (Giancotti and Tarone, 2003). Rac exchange factors Vav1 and Vav2 are substrates of Syk/ZAP70 and once active, Vav1 recruits Sos, thus providing a link to the MAPK pathway (Reynolds et al., 2004).

The seventh mechanism by which integrins activate the MAPK pathway is through protein kinase C (PKC). PKC is known to activate Raf, thus providing a link to the MAPK pathway (Giancotti and Ruoslahti, 1999, Bjorkoy, 1995 #97). It has been reported that upon phosphatidylcholine (PC) hydrolysis, elevated levels of PC-derived 1,2 diacylglycerol (DAG) result in PKC-ζ mediated Raf activation (Bjorkoy et al., 1995). In addition, phosphoinositide 3 kinase (PI-3K) has been implicated in activating PKCζ, which then activates Raf (Giancotti and Ruoslahti, 1999; Mas et al., 2003). Thus PKC-mediated activation of the MAPK pathway through Raf has been reported through two distinct pathways.

Importantly, while there are many pathways by which integrins can potentially activate the canonical MAPK pathway, not all of these pathways are firmly established (Giancotti and Ruoslahti, 1999; Giancotti and Tarone, 2003; Howe et al., 2002; Lee and Juliano, 2004; Mas et al., 2003; Miranti et al., 1998). In addition, these pathways may not all exist in every cell line and for every integrin (Giancotti and Tarone, 2003; Miranti et al., 1998). Furthermore, integrin-mediated stimulation has also been shown to activate pathways involving Rho (Danen et al., 2000), a GTPase that has been firmly established as a cytoskeleton regulator (Ridley and Hall, 1992), and thus provides a possible link

between cell morphology and Erk activation. Additionally, RhoA has been found to activate MAP/Erk kinase kinase 1 (MEKK1) in yeast, providing a link between Rho and activation of Erk (Chen and Cobb, 2006)

3.2 *Deactivation mechanisms*

Erk is also concurrently regulated by deactivation machinery. Deactivation pathways can be loosely grouped into one of three motifs. In the first motif, deactivation occurs without stimulus; because this form of deactivation is present in the absence of any other stimulation, it is termed as constitutive direct-deactivation. As the signal activation reaction is not an irreversible chemical reaction, an equilibrium reaction exists between the active phosphorylated state and the inactive dephosphorylated state. Thus, deactivation due to dephosphorylation of Erk by either dissociation of the active phosphorylated signal to an inactive dephosphorylated form due to equilibrium or via basal phosphatase activity may occur (Keyse, 2000; Lewis et al., 1998; Tamura et al., 2002).

In the second motif, active Erk initiates a signaling pathway that ultimately leads to transcription of phosphatase(s) that target its deactivation (Brondello et al., 1997; Grumont et al., 1996). Because in this form of deactivation, active Erk essentially targets itself, it is referred to as feedback-mediated direct deactivation. Activation of Erk has been observed to regulate map kinase phosphatases (Mkp) through both an upregulation of the rate of phosphatase transcription (Brondello et al., 1997) and a reduction in the rate of proteosome-mediated degradation (Brondello et al., 1999). Activation of Erk has been

found to induce transcription of Mkp1 and Mkp2 (Brondello et al., 1997). In addition, active Erk has also been observed to upregulate Mkp3 via Erk-mediated binding to its regulatory domain (Camps et al., 2000; Camps et al., 1998). Erk-mediated phosphorylation of Mkp1 on Serine-359 and Serine-364 protects it from proteosome-mediated degradation (Brondello et al., 1999). In addition, adhesion-mediated regulation of phosphatases have also been observed. For example, the cytoplasmic tail of α_1 integrin selectively interacts with the ubiquitously expressed T-cell protein tyrosine phosphatase (TCPTP) and activates it after adhesion to collagen. (Mattila et al., 2005). Several addition candidates for Erk-mediated phosphatase upregulation are PAC-1, *vaccinia* H1-related (VHR), and haemopoietic protein tyrosine phosphatase (HePTP), all of which are known to act on Erk (Keyse, 2000).

In the final deactivation motif, active Erk deactivates one of its upstream activators via hyperphosphorylation (Brunet et al., 1994; Buday et al., 1995; Cherniack et al., 1995; Dong et al., 1996; Langlois et al., 1995; Wartmann et al., 1997). This form of deactivation is termed feedback-decoupling deactivation. In the Erk signaling pathway, feedback-decoupling deactivation is mediated by both adaptor-targeted feedback (Buday et al., 1995; Cherniack et al., 1995; Dong et al., 1996; Langlois et al., 1995) and enzyme-targeted feedback (Brunet et al., 1994; Wartmann et al., 1997). In adaptor-targeted feedback, Sos complexed with Grb2 undergoes serine/threonine hyperphosphorylation. Once hyperphosphorylated, the complex either dissociates from the active receptor (Buday et al., 1995) or separates back into Sos and Grb2 (Cherniack et al., 1995; Dong et al., 1996; Langlois et al., 1995), both of which render hyperphosphorylated Sos to an

inactive state. Both Erk, and an enzyme downstream of Erk have been implicated as responsible for Sos hyperphosphorylation (Anderson et al., 1991; Corbalan-Garcia et al., 1996). In enzyme-targeted feedback, Raf (Wartmann et al., 1997), Mek (Brunet et al., 1994), and Fak (Hunger-Glaser et al., 2003) hyperphosphorylation have been shown to trigger signal attenuation. Consistent with both these findings, hyperphosphorylation of Mek on Thr^{292} and Thr^{386} (Brunet et al., 1994) and Raf (Brunet et al., 1994; Wartmann et al., 1997) on serine residues 29, 43, 289, 296, 301, and 642 have been implicated as substrates for Erk (Dougherty et al., 2005). Hyperphosphorylation of Fak on Serine-910 is believed to be mediated either via Erk or protein kinase C (Hunger-Glaser et al., 2003). Recently, the hyperphosphorylated form of Raf has been observed to return to an active signaling state through interaction and dephosphorylation with the serine/threonine phosphatase PP2A and the prolyl isomerase Pin1 (Dougherty et al., 2005). Although phosphatases that associate with and activate the inactive hyperphosphorylated forms of Mek and Fak via dephosphorylation have not been determined, serine/threonine phosphatases that can target many different substrates, such as PP2A, may act to catalyze the activation of the inactive hyperphosphorylated forms of Mek and/or Fak.

4. Model Description

4.1 *Coarse-grain model*

A simple mathematical model which lumps Erk activation and deactivation mechanisms into four distinct biological motifs is depicted in Figure IV-2. In this model, the input stimulus (I) activates the signal (S), converting it to its active form (S*) as

shown in motif 1, which we term simply as 'activation.' The signal S in our model refers to Erk. Active signal (S*) can be deactivated in the absence of any other stimulation as illustrated in motif 2, which we term 'constitutive direct-deactivation.' Deactivation can also occur via feedback mechanisms. In the first feedback mechanism, active signal (S*) deactivates itself as shown in motif 3, which we term 'feedback-mediated direct-deactivation.' In the second feedback mechanism, feedback occurs by converting input stimulus (I) to a permanently inactive form (I-) as shown in motif 4, which we term 'feedback decoupling deactivation.'

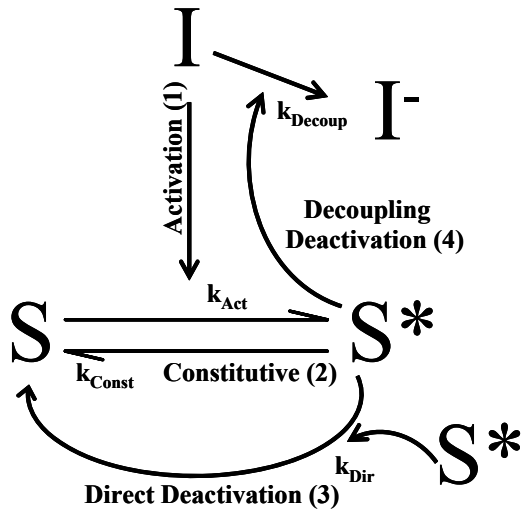

Figure IV-2: Model schematic
The input stimulus (I) activates the signal (S), converting it to its active form (S*) as shown in motif 1, termed simply as 'activation.' Active signal (S*) can be deactivated in the absence of any other stimulation as illustrated in motif 2, termed 'constitutive direct-deactivation.' Deactivation can also occur via feedback mechanisms. In the first feedback mechanism, active signal (S*) deactivates itself as shown in motif 3, termed 'feedback-mediated direct-deactivation.' In the second feedback mechanism, feedback occurs by converting input stimulus (I) to a permanently inactive form (I-) as shown in motif 4, termed 'feedback-decoupling deactivation.' In each of these motifs, it is assumed that the kinetics of each pathway can be described by a single rate constant.

In each of these motifs, it is assumed that the kinetics of each pathway can be described by a single rate constant. Thus, the rate is proportional to the species concentration connected by each arrow. In the decoupling motif, it is assumed that permanent deactivation of (I) occurs over the time course of the experiment. While the first assumption may not be valid if non-linear relationships exist between the connected species and the second assumption may not be valid at late times, both are reasonable starting postulates for a coarse-grain model.

4.2 *Activation motif (motif 1)*

The activation rate constant k_{Act} incorporates both growth factor- and integrin-mediated activation pathways when appropriate to the system of study. Growth factor-mediated Erk activation contributions to k_{Act} are due to growth factor-induced receptor activation that enables formation of Sos-Grb2 (Roovers and Assoian, 2000). Recall that this allows Ras to be activated and initiates a kinase cascade resulting in Erk activation (Roovers and Assoian, 2000). Likewise, adhesion-mediated Erk activation contributions to k_{Act} will involve aforementioned integrin mediated pathway(s) leading to subsequent Erk activation (Bjorkoy et al., 1995; Giancotti and Ruoslahti, 1999; Hanks and Polte, 1997; Howe et al., 2002; Mas et al., 2003; Miranti et al., 1998; Schlaepfer et al., 1994; Vuori, 1998). Note that the relative importance of these pathways cannot be determined by the model at this stage of development.

4.3 *Constitutive direct-deactivation (motif 2)*

Because constitutive direct-deactivation is independent of feedback control, it is written as a reversible step in the model schematic (Figure IV-2). Contributions from constitutive phosphatase activity as well as dissociation of the active phosphorylated form of Erk to its inactive dephosphorylated form are incorporated into k_{const}. For the Erk activation pathway, constitutive phosphatases of importance may include any of the aforementioned phosphatases known to act on Erk (Keyse, 2000; Tamura et al., 2002).

4.4 *Feedback-mediated direct-deactivation (motif 3)*

In feedback-mediated direct-deactivation, the active signal (S*) initiates deactivation of itself, thus this deactivation motif is a form of feedback control. For the Erk signaling pathway, direct-deactivation feedback occurs when active Erk initiates a signaling pathway that ultimately leads to the transcription of phosphatase(s) that target its deactivation (Brondello et al., 1997; Grumont et al., 1996). All forms of feedback-mediated direct-deactivation acting on the cell are incorporated into k_{dir}.

4.5 *Feedback-decoupling deactivation (motif 4)*

Feedback-decoupling deactivation is an activation-targeting form of feedback control where the active signal S* acts on the upstream species I and renders it permanently inactive by changing it to I^-. As mentioned previously, in the Erk signaling pathway feedback-decoupling occurs when active Erk permanently deactivates growth factor receptor (Matveev and Smart, 2002), Sos (Buday et al., 1995; Chen et al., 1996; Cherniack et al., 1995; Corbalan-Garcia et al., 1996; Langlois et al., 1995), Raf

(Wartmann et al., 1997), Mek (Brunet et al., 1994), or Fak (Lee and Juliano, 2004) via hyperphosphorylation. All forms of feedback-decoupling deactivation present in the cell are incorporated into $k_{decoupl}$.

4.6 *Model equations*

From the model schematic pictured in (Figure IV-2), differential equations describing the signal and input kinetics are shown in Equation 1 below.

$$\frac{d[S^*]}{dt} = k_{Act}[S][I] - k_{Const}[S^*] - k_{Dir}[S^*][S^*]$$
$$\frac{d[I]}{dt} = -k_{Decoup}[S^*][I] \qquad \textbf{(Equation 1)}$$

The following mass balances for signal (S) and input (I) apply (Equation 2):

$$[S_o] = [S] + [S^*]$$
$$[I_o] = [I] + [I^*] \qquad \textbf{(Equation 2)}$$

Dimensionless parameters are defined as:

$$\tau = \frac{t}{t_o} \qquad \theta_S = \frac{[S^*]}{[S_o]} \qquad \theta_I = \frac{[I]}{[I_o]} \qquad \textbf{(Equation 3)}$$

Substituting the mass balances (Equation 2) and dimensionless parameters (Equation 3) into the model equation (Equation 1), the dimensionless form of the model is written:

$$\frac{d\theta_S}{d\tau} = \kappa_{Act}(1-\theta_S)\theta_I - \kappa_{Const}\theta_S - \kappa_{Dir}\theta_S^2$$
$$\frac{d\theta_I}{d\tau} = -\kappa_{Decoup}\theta_S\theta_I \qquad \textbf{(Equation 4)}$$

Where the dimensionless groups are defined in Equation 5

$$\kappa_{Act} = k_{Act}[I_o]t_0 \qquad \kappa_{Const} = k_{Const}t_0$$
$$\kappa_{Dir} = k_{Dir}[S_o]t_0 \qquad \kappa_{Decoup} = k_{Decoup}[S_o]t_0 \qquad \textbf{(Equation 5)}$$

5. Results and Discussion

5.1 *Model results*

We begin by examining the effect of the activation motif on signaling kinetics in the absence and presence of each form of deactivation. Deactivation motifs are first studied separately in order to determine the individual effect of each on temporal profiles. Once the contributions of individual motifs are known, we consider the combined effect of multiple forms of deactivation on signal dynamics.

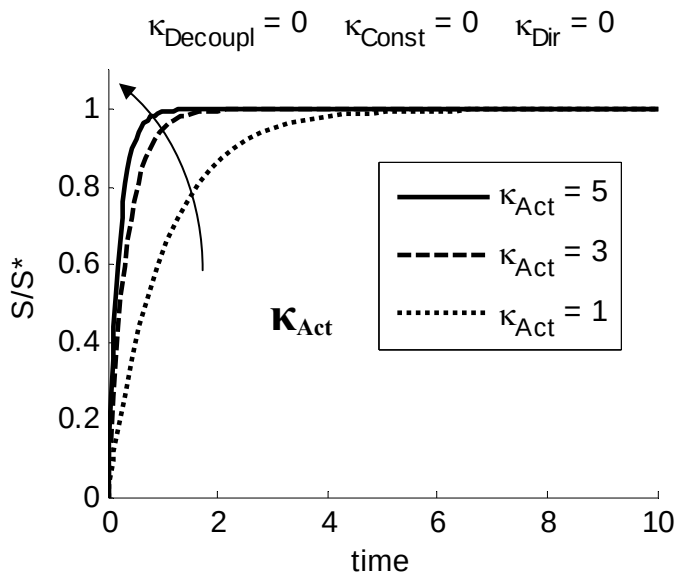

Figure IV-3: Activation alone induces sustained signal activation.
The effect varying the activation dimensionless group (κ_{Act}) on signaling kinetics.

In the absence of any deactivation mechanism, the rate at which active signal (S*), reaches its maximum value is determined by the value of the dimensionless activation rate constant, κ_{Act}. As the value of this dimensionless parameter is increased, the time taken to achieve maximum intensity decreases (Figure IV-3). Note that the signal intensity is limited between zero and unity due to dimensionalization.

When activation is coupled with only one form of deactivation, a sustained signal is observed regardless of the type of deactivation (Figure IV-4). Although all three deactivation dimensionless groups affect signaling kinetics similarly, with signal maximum decreasing with increasing dimensionless deactivation constant, each motif affects the maximum signal intensity differently (Figure IV-4). As can be seen from Figure IV-4, increasing constitutive direct-deactivation has the greatest impact on signal

reduction, while increasing feedback decoupling deactivation has the least effect (compare Figure IV-4A, to Figure IV-4B, to Figure IV-4C).

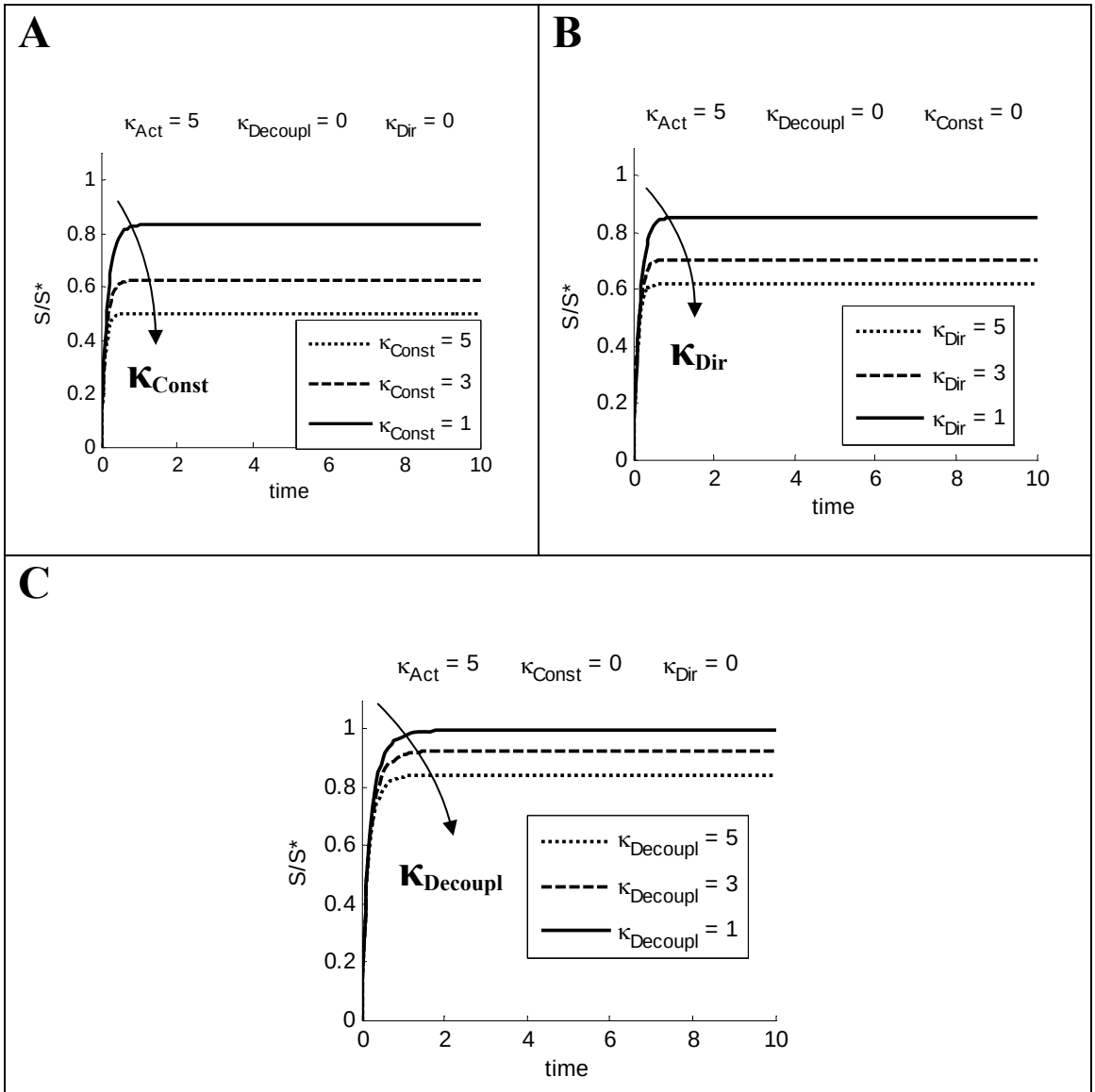

Figure IV-4: Effect of each form of deactivation on signal activation kinetics.

The effect of activation and only one form of deactivation was investigated. Dimensionless groups for (A) constitutive direct-deactivation (κ_{const}), (B) feedback-mediated direct-deactivation (κ_{dir}), and (C) feedback-decoupling deactivation ($\kappa_{decoupl}$) were varied to determine the effect of each on signaling kinetics.

When activation and all three forms of deactivation are present, a transient signal is observed (Figure IV-5). Because no transient activation is observed in the presence of a single deactivation motif (Figure IV-4), a transient signal requires multiple forms of deactivation. Furthermore, we find that all three motifs of deactivation are not necessary to obtain a transient signal. Our model demonstrates that the minimal requirement for transient signaling is that feedback decoupling deactivation must be complemented by at least one form of direct-deactivation (Figure IV-6, compare A to B and C).

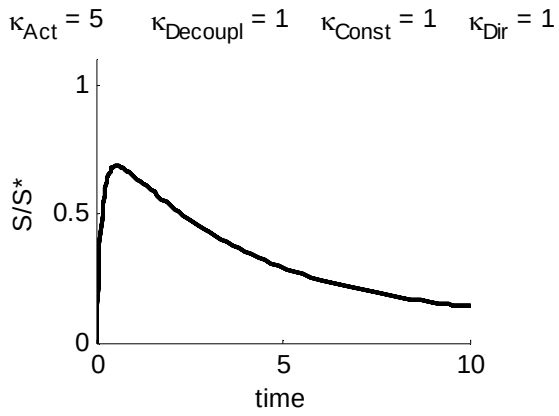

Figure IV-5: Activation and all three forms of deactivation results in transient signal activation.

A transient signal is observed when activation and all three forms of deactivation are present.

Analysis of Figure IV-5 and Figure IV-6 illustrates that activation, feedback-decoupling deactivation, and one form of direct-deactivation are required for establishment of a transient signal. Therefore, we begin our detailed analysis of transient signaling kinetics by investigating the importance of the magnitude of activation on the observed transient profile. Following this analysis, the effects of deactivation are

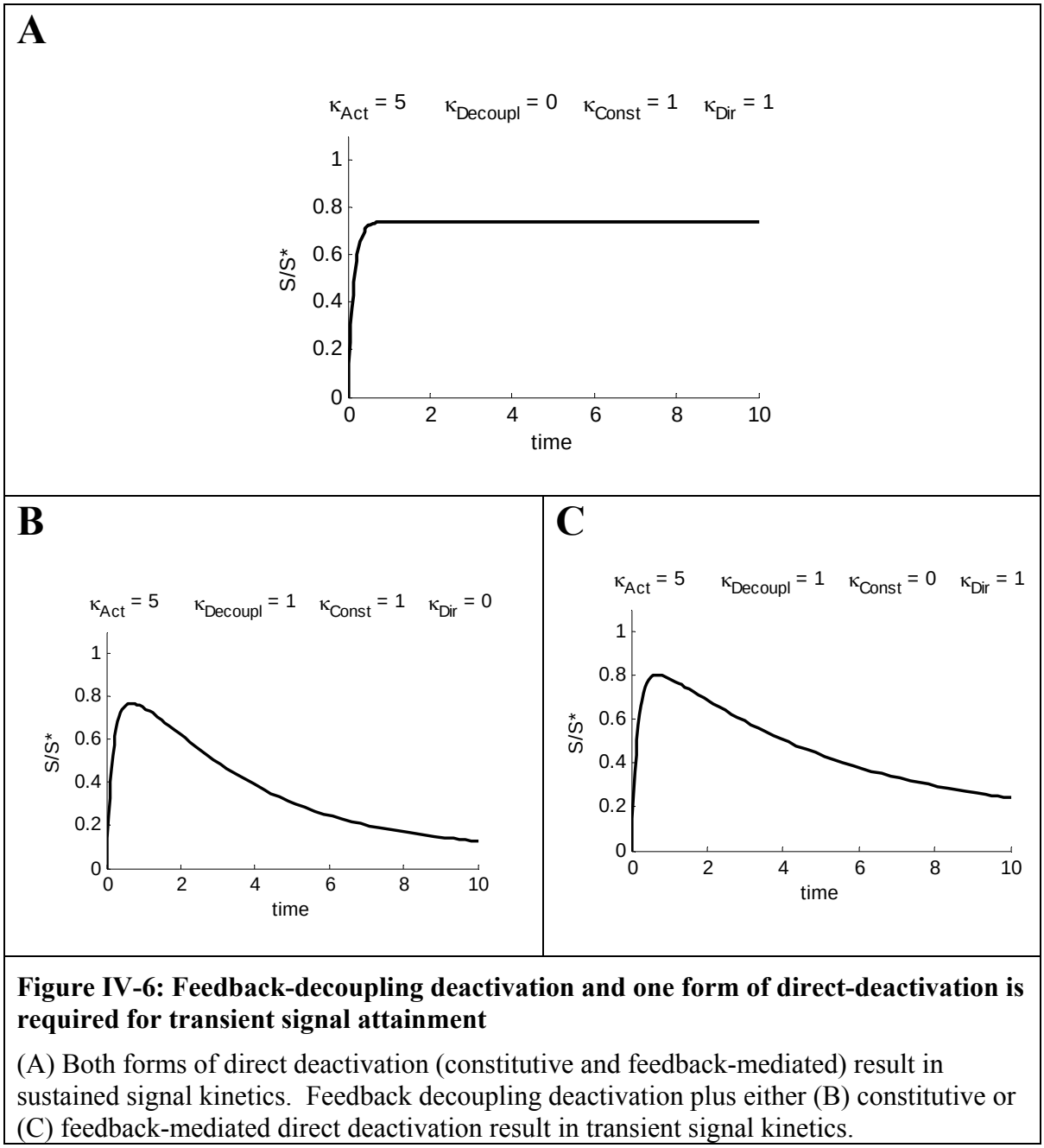

Figure IV-6: Feedback-decoupling deactivation and one form of direct-deactivation is required for transient signal attainment

(A) Both forms of direct deactivation (constitutive and feedback-mediated) result in sustained signal kinetics. Feedback decoupling deactivation plus either (B) constitutive or (C) feedback-mediated direct deactivation result in transient signal kinetics.

investigated, starting with feedback decoupling. Recall that in addition to feedback-decoupling deactivation, one form of direct-deactivation is also required for attainment of a transient signal. Thus, two distinct cases are investigated that include activation and feedback-decoupling deactivation either with (1) constitutive direct-deactivation as the

required form of direct-deactivation, or (2) feedback-mediated direct-deactivation as the necessary form of direct-deactivation. To facilitate comparison between each of these cases across analyses, a common profile with $\kappa_{act} = 5$, $\kappa_{decoupl} = 1$ and $\kappa_{direct\_deactivation} = 1$ (where the form of direct deactivation is either constitutive or feedback-mediated) is provided for reference in each of the graphs presented, and this common profile always appears as a solid line. Thus, the relative effect of varying each motif independently can be determined by comparing the reference (solid line) provided for each case. Note that the reference is not equivalent across cases as the common profiles are not equivalent when different forms of direct deactivation are present.

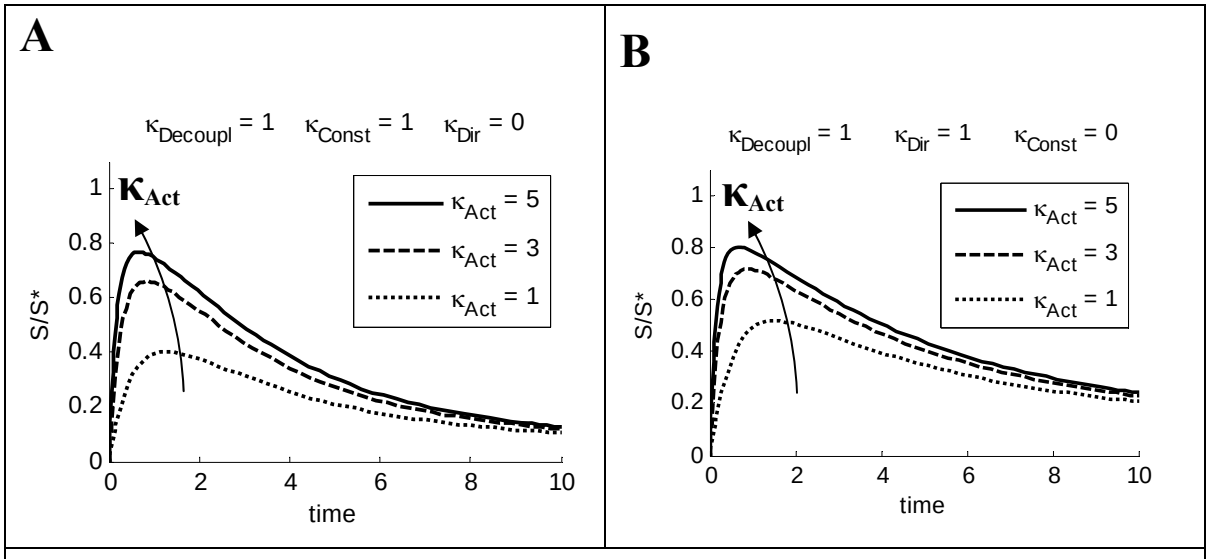

Figure IV-7: The effect of varying activation on transient profiles

Varying activation results in an increase in peak signal magnitude regardless of whether the required form of direct-deactivation is due to (A) constitutive or (B) feedback-mediated direct-deactivation

We find that identical trends are observed in the resultant transient profile when activation is varied regardless of which form of direct-deactivation is present. In either case, increasing the magnitude of the activation dimensionless group increases the

maximum peak intensity and the time at which signal returns to basal is not affected (Figure IV-7A).

As feedback-decoupling is the only form of deactivation that is required for establishment of a transient signal, we begin our analysis of the effect of deactivation on transient signaling kinetics by investigating the importance of the magnitude of feedback-decoupling deactivation on the observed transient signaling profile. Again, two distinct cases are investigated: activation and feedback-decoupling deactivation in the presence of either constitutive (Figure IV-8A) or feedback-mediated (Figure IV-8B) direct-deactivation.

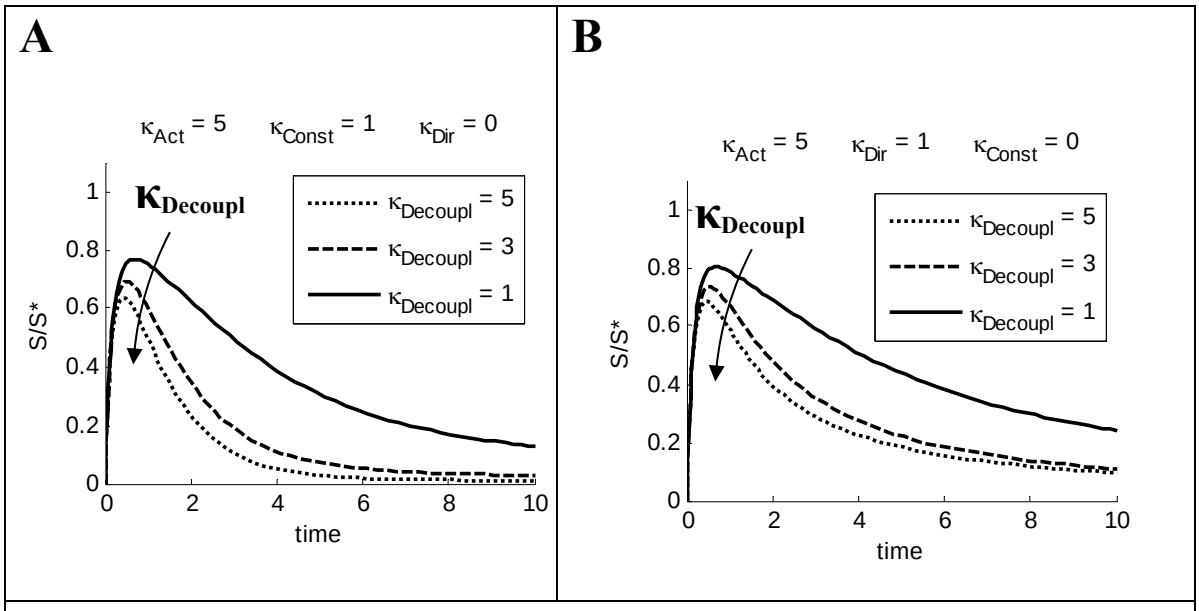

Figure IV-8: The effect of varying feedback-decoupling deactivation on transient profiles

Varying decoupling deactivation results in depression of the maximum signal intensity and acceleration of the signal return to basal regardless of whether the required form of direct-deactivation is due to (A) constitutive or (B) feedback-mediated direct-deactivation.

We again find that the observed trends are the same, regardless of which form of direct-deactivation is present. When the feedback-decoupling deactivation dimensionless group is increased, peak signal intensity is depressed and the return to basal levels is accelerated (Figure IV-8).

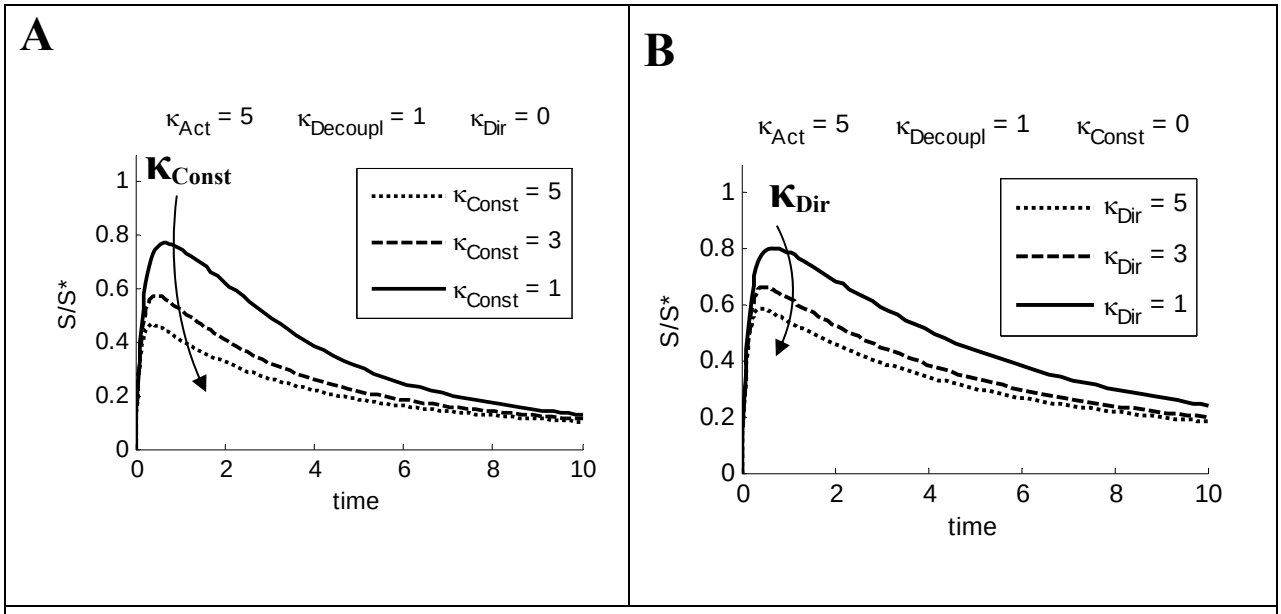

Figure IV-9: The effect of varying direct-deactivation on transient profiles

Increasing the direct-deactivation dimensionless group results in peak signal magnitude depression without affecting the signal's return to basal signal level regardless of whether the required second form of direct- deactivation is due to (A) constitutive or (B) feedback-mediated direct-deactivation.

In order to more fully understand how the direct-deactivation motifs affect signaling kinetics, the dimensionless group for either constitutive or feedback-mediated direct-deactivation was varied independently in the presence of the other two required motifs: activation and feedback-decoupling deactivation. Increasing either form of

direct- deactivation results in depressing the signal peak intensity without affecting the signal's return to basal level (Figure IV-9).

Our model reveals that feedback-decoupling deactivation and one form of direct-deactivation are both necessary and sufficient for establishment of a transient signal (Figure IV-6). Observed trends for activation and feedback-decoupling are similar regardless of which form of direct-deactivation (constitutive or feedback) is present. Furthermore, when the magnitude of the activation dimensionless group is increased, our model predicts that an increase in signal peak magnitude will occur without affecting the time at which signal returns to basal (Figure IV-7A). In contrast, when the feedback decoupling deactivation dimensionless group is increased, peak signal intensity is depressed and the return to basal levels is accelerated. In addition, we find that increasing direct-deactivation (either constitutive or feedback-mediated) results in depressing the signal peak intensity without affecting the signal's return to basal levels. Finally, our model indicates that the constitutive form direct-deactivation has a greater impact on signal kinetics than does feedback-mediated direct-deactivation, regardless of which parameter is being varied.

5.2 *Comparison to experimental results*

We use the results of the model to focus the search for potential mechanisms responsible for experimentally observed Erk activation profiles. The model is first used to focus the search for molecular mechanisms responsible for the observed adhesion-induced enhancement EGF-mediated Erk signaling. The model is then later used to

investigate potential mechanistic explanations for the observed differences in signaling kinetics in adherent cells stimulated by each of the three growth factors (EGF, PDGF, or bFGF).

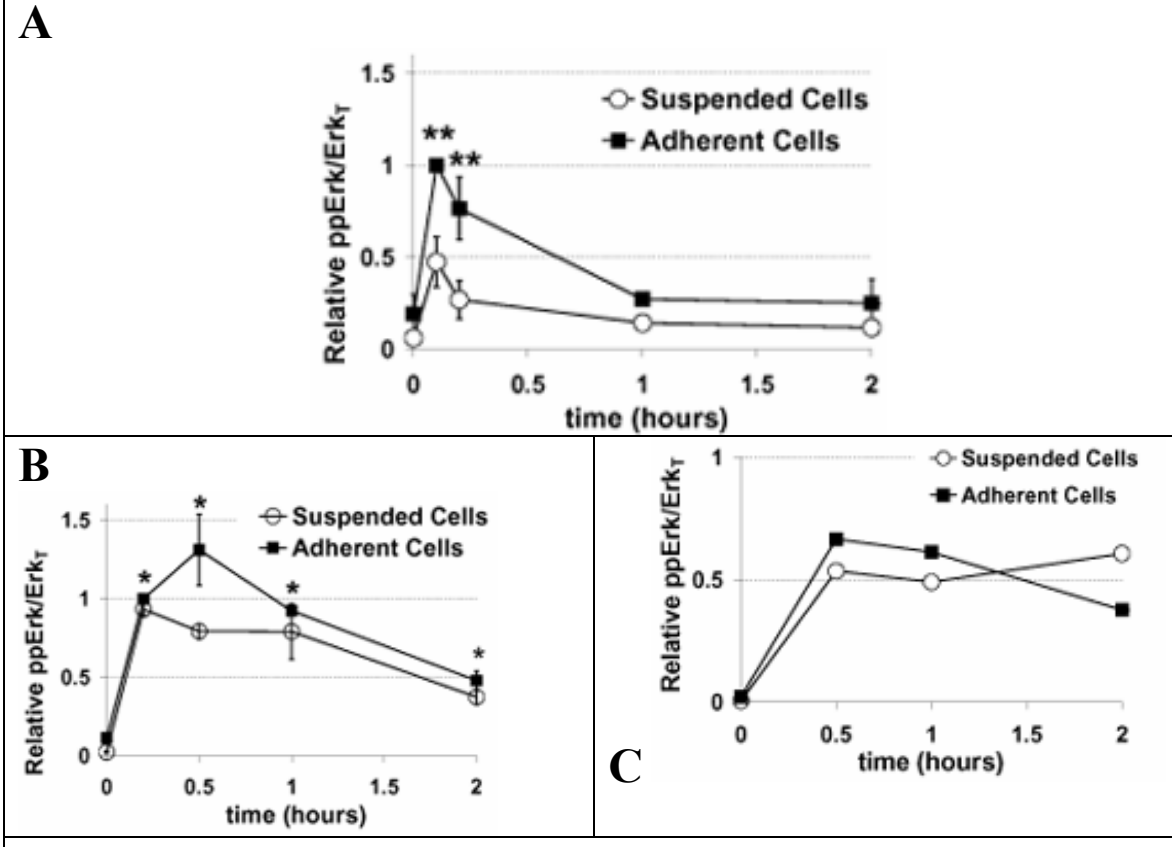

Figure IV-10: Experimental Erk activation profiles

A single asterisk denotes that Erk activation in the adherent and suspended profiles is statistically equivalent, while a double asterisk denotes the two profiles are statistically different using Student's t-test. (A) EGF-mediated Erk activation is transient for both adherent and suspended cells. Cells were stimulated with 800 pM EGF for the indicated times. (B) PDGF induces transient Erk activation in adherent cells. Cells were stimulated with 800 pM PDGF for the indicated times. (C) bFGF induces sustained activation of Erk. Cells were stimulated with 1000 pM bFGF for the indicated times prior to lysing. The graph represents a single experiment.

The results of our experiments demonstrate that EGF clearly induces a transient activation of Erk in both adherent and suspended cells. In both cases, the signal returns to basal within one hour following stimulation (Figure IV-10A). Classification of PDGF-mediated Erk activation in adherent cells requires use of our definitions for transient and sustained. Note that PDGF-mediated Erk activation in adherent cells peaks and steadily decreases to one-third of the maximal signal intensity. Therefore, according to our definition that a sustained signal peaks and maintains a signal that is equal to at least half-maximum, PDGF-stimulation also induces a transient activation of Erk (Figure IV-10B). In contrast, bFGF-mediated Erk activation in adherent cells is indubitably sustained (Figure IV-10C) as the signal remains at maximum signal intensity throughout the time course.

The model is first used to narrow down potential mechanism(s) responsible for the observed enhancement of EGF-mediated Erk activation in adherent cells as compared to suspended cells. Note that for adherent and suspended cells stimulated via EGF, the early phase of Erk activation (< 1 h) is adhesion-dependent, while the late phase of the signal reaches a nearly equivalent, basal signal for both adhered and suspended cells (Figure IV-10A). In addition, both the adherent and suspended cells reach maximum signal intensity rapidly (Figure IV-10A). One possible explanation is that the magnitude of the activation dimensionless group is greater in the adherent cells than in the suspended cells (Figure IV-7). As adhesion is known to induce Erk activation by mechanisms distinct from growth factors, an increase in the activation dimensionless group for cells adhered to fibronectin is a likely explanation. However, the model also reveals that differences in either of the two required forms of deactivation required for

transient signal attainment could be responsible for the observed difference in signal peak magnitude (Figure IV-8 and Figure IV-9). As the possibility that adhesion reduces negative feedback cannot be discounted, both differences in activation and deactivation mechanisms may occur.

The model is then used to predict potential mechanisms explaining dynamic differences resulting from differential growth factor stimulation. As the model incidated that only the presence of feedback-decoupling deactivation and one form of direct-deactivation result in transient profile attainment (Figure IV-5; Figure IV-6, A-C), these motifs must be present in both EGF- or PDGF-mediated stimulation of adherent cells (Figure IV-6, A-C). In addition, the model predicts that for bFGF simulation of adherent cells, either no deactivation (Figure IV-3), any single form of deactivation (Figure IV-4), or both forms of direct-deactivation (Figure IV-6A) may be responsible for the observed sustained kinetic profile.

6. Conclusions

A simple coarse-grain mathematical model was developed to focus the search for molecular mechanism(s) driving the magnitude and kinetics of experimentally observed Erk activation profiles. However, it should be noted that the model is not restricted to investigation of Erk activation, but rather, can be applied to any system in which the four identified motifs are present. The model demonstrates that activation, feedback-decoupling deactivation, and one form of direct-deactivation are required to generate a

transient signal. Interestingly, the form of direct-deactivation (either constitutive or feedback-mediated) has no effect on the observed signaling trend. We also find that increasing the magnitude of the activation dimensionless group increases the maximum signal peak and the time at which signal returns to basal is not affected, regardless of which form of direct-deactivation is present. Notably, the activation dimensionless group has the most significant affect on peak signal magnitude. In contrast, when the feedback-decoupling deactivation dimensionless group is increased, peak signal intensity is depressed and the return to basal levels is accelerated irrespective of which form of direct-deactivation is present. Lastly, we find that increasing either required form of direct-deactivation (constitutive or feedback-mediated) results in signal peak intensity depression without affecting the signal's return to basal level. Interestingly, in all cases, the signaling kinetics are most sensitive to variation of any single motif's dimensionless group when constitutive direct-deactivation is the required form of direct-deactivation, instead of feedback-mediated direct-deactivation being present.

Our model results are used to narrow the search for the molecular mechanisms responsible for our experimental observations detailed in Chapter 3. Recall that our experimental results demonstrate that EGF-stimulation clearly induces transient Erk activation in both adherent and suspended cells, and that this EGF-mediated Erk activation is adhesion-dependent. Furthermore, a comparison of adherent and suspended experimental activation profiles reveals that adherent and suspended cells exhibit similar signaling dynamics, but differ in maximum peak intensity with signal magnitude being enhanced in adherent cells. Thus, our model suggests the most likely mechanism

responsible for adhesion-dependent Erk activation is an increase in the activation rate constant upon adhesion. Alternatively, the model also indicates decreasing any deactivation dimensionless group can enhance signal magnitude, but not to nearly as great as an extent as that resulting from an increased activation rate.

Model results can also be used to focus the mechanistic search for the intriguing differences in Erk activation dynamics resulting from stimulation of adherent cells by distinct growth factors. Recall that the model predicts that decoupling deactivation must be present in cells stimulated by either PDGF or EGF due to the generation of a transient Erk activation signal. If we discover decoupling deactivation occurs in bFGF-stimulated adherent cells, the model predicts direct-deactivation cannot be present in these cells and still produce sustained Erk activation. Thus, in this case, the presence of direct-deactivation in PDGF- and EGF-stimulated adherent cells, and its absence in bFGF-stimulated adherent cells, would be the mechanism responsible for differences in Erk activation dynamics. However, if no feedback-decoupling deactivation is found to occur in adherent cells stimulated by bFGF, the model predicts that any form(s) of direct-deactivation result in the observed sustained Erk activation. Thus, in this case it would be the presence of feedback-decoupling in PDGF- and EGF-stimulated adherent cells, and its absence in bFGF-stimulated adherent cells, that would be the mechanism responsible for the observed differences in Erk activation dynamics.

We use the results of this simple coarse-grain mathematical model to elucidate how specific molecular mechanisms contribute to the dynamics of Erk signaling. Model

results are then compared to our experimentally determined Erk signaling profiles (Chapter 3) to infer potential mechanisms and to direct future experiments. To that end, both the significance of our findings and the future work suggested by our experiment results (Chapter 3) and model predictions (Chapter 4) will be discussed in detail in Chapter 5.

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Chapter V. Conclusions and Future Work

1. Significance of Findings

This study demonstrates that cell adhesion has quantitatively intricate effects on growth factor-mediated Erk signaling. We reported that the effect of cell adhesion is specific to the type of growth factor, its dose, and the timing of stimulation. Our system utilizes a single cell line, and each study is subjected to identical experimental conditions prior to growth factor stimulation to ensure that valid comparisons and conclusions are drawn. We find that adhesion to FN selectively enhances Erk signaling elicited by EGF, but has no effect on bFGF- or PDGF-mediated Erk activation. Unexpectedly, at concentrations of PDGF and bFGF that are significantly less than the dissociation constant, cell adhesion severely attenuates growth factor-mediated Erk signaling. Thus, adhesion not only enhances cell response to specific growth factors, but also filters out potentially noisy signals from low levels of growth factor.

Our study reveals that cell adhesion is not always a positive effector of signal transduction and that, surprisingly, cell adhesion can negatively affect signaling. Thus, cell adhesion can no longer be viewed exclusively as a positive activator of signaling. Instead, we must adjust our view of adhesion; adhesion can act as either a positive or negative regulator of activation. Importantly, this change in perspective will be significant in the area of biomaterial development as researchers must now consider the possibility that enhancing adhesivity could inadvertently quiesce the very signals they are trying to augment. Such considerations will be especially important in selecting adhesive

epitopes to integrate into biomaterial surfaces. The potential for adhesion to negatively regulate signal activation also suggests researchers will need to develop assays to ascertain whether or not they have accidentally incorporated this negative effect into the biomaterial surface under development. To that end, our findings raise the question of whether we can decouple the mechanisms of adhesion-mediated negative and positive regulation on signal activation so that biomaterial surfaces can be developed which incorporate only the desirable positive effects of adhesion on signal activation.

In addition, our study begins to provide a systematic comparison of the crosstalk between adhesion and three different growth factors. Deciphering these and other quantitatively intricate ways in which cell adhesion influences growth factor signaling will be crucial to developing a better understanding of how the adhesive microenvironment 'primes' cell behaviors. Such quantitative insight will be important in designing synthetic microenvironments for applications such as tissue engineering and regenerative medicine, where rigorous quantification will be required. In addition, quantification will enable improved assessment of the quality and effectiveness of both biomaterials and cancer therapies.

2. Overview of Future Research

Future work will focus on uncovering the molecular mechanisms governing the three most significant experimental results: (1) adhesion-mediated desensitization of signal activation by selective growth factors, (2) adhesion-dependent Erk activation

enhancement by EGF stimulation, and (3) growth-factor specific signaling dynamics of Erk activation in adherent cells. Investigation into potential pathways contributing to the observed Erk signaling kinetics may have a bilateral approach; either experimental data independently or experimental data in combination with model predictions may be used to guide future experiments. Experimental results may be used to design additional new experiments to ascertain which upstream activator is responsible for adhesion-dependent growth factor-mediated desensitization of Erk activation. In contrast, experimental data in conjunction with model results may be used to focus the mechanistic search for both EGF-mediated adhesion-dependent Erk activation and growth-factor specific Erk signaling dynamics in adherent cells.

3. Adhesion-Mediated Desensitization of Signal Activation by Selective Growth Factors

3.1 *Background*

Our experimental work discussed in detail in Chapter 3 demonstrates that at critical sub-saturating doses of PDGF or bFGF, adhesion to fibronectin actually desensitizes the cell to growth factor stimulation, rendering the signaling pathway unresponsive to growth factor. Our experimental findings are particularly interesting given that adhesion is historically considered as a positive effector. However, while adhesion-mediated desensitization is a novel finding, there are many examples of Erk signaling desensitization in the literature, where exposure to a growth factor desensitizes the cell from signal activation when exposed to a second charge of the same and/or different growth factor.

As the hyperphosphorylation of Sos in response to growth factor stimulation has been found to be a prominent mechanism by which growth factor desensitizes Erk signaling (Chen et al., 1996; Cherniack et al., 1995; Corbalan-Garcia et al., 1996; Langlois et al., 1995), we began our search for the mechanism of adhesion-mediated desensitization of Erk activation to EGF/PDGF-stimulation by assaying Sos hyperphosphorylation. However, experimental results clearly demonstrate that adhesion to FN does not induce Sos hyperphosphorylation in our system (Figure III-6, III-16). Thus, the adhesion-mediated desensitization we observe does not occur at the level of Sos regulation.

To focus our search for the mechanism responsible for adhesion-induced desensitization, we varied the 'priming time,' or duration of adhesion prior to growth factor stimulation, and found that priming time was critical to the desensitization process. Interestingly, Erk and Akt activation significantly improved only when pre-exposure to adhesion was completely eliminated and concurrent stimulation of adhesion and growth factors occurred. However, concurrent stimulation by growth factor and adhesion was only able to partially rescue adhesion-mediated desensitization of PDGF- and bFGF-mediated Erk and Akt signaling. The fact that concurrent stimulation was not able to fully rescue adhesion-mediated desensitization to PDGF or bFGF suggests that the time scale of desensitization is rapid. Moreover, because both Ras and growth factor receptors are known to be upstream activators of Akt (Downward, 2004; Shaw and Cantley, 2006), these findings further suggest that adhesion-mediated desensitization targets a regulatory

point upstream of Ras and proximal to growth factor receptor activation. Thus, although Sos is not the target of desensitization, another signal proximal to growth factor detection must be involved.

In fact, several growth factor receptors directly interact with adhesion receptors (Comoglio et al., 2003). While the association of growth factor receptors with adhesion receptors has been predominantly correlated with positive synergism, it may also sequester and inhibit the activity of low levels of ligand-bound growth factor receptors. Indeed, such heterologous desensitization by receptor sequestration has been demonstrated for EGFR and PDGFR via stimulation by PDGF or EGF (Matveev and Smart, 2002). Hence, there is precedent for desensitization to selectively affect a subset of growth factors (Hupfeld et al., 2005; Matveev and Smart, 2002).

3.2 *Future work*

To test if regulation occurs at the level of growth factor receptor, phosphor-tyrosine blots may be run for both adherent and suspended cells stimulated with either PDGF or bFGF using the experimental system we developed. If differences in growth factor receptor tyrosine phosphorylation levels are observed over the time course, this indicates that some form of regulation occurs at the level of growth factor receptor. To investigate this mechanism further, development of an adhesion-mediated receptor sequestration assay, possibly adapting the solid-phase binding assay protocol outlined by Wijelath and colleagues (Wijelath et al., 2002), would be required. If growth factor

receptor sequestration levels cannot explain regulation at the level of receptor, alternate mechanisms would have to be investigated to explain the observed result.

One such possible mechanism may involve direct interactions between growth factors and ECM proteins. Sequestration of TGFβ, VEGF, and HGF by ECM is well documented (Griffith and Swartz, 2006). In fact, VEGF has recently been shown to bind specific sites on FN, suggesting potential sequestration in more basic reconstituted systems. Thus, ECM protein-mediated sequestration may play a role in diminishing PDGF- or bFGF-mediated signaling on FN-coated dishes, especially in systems employing low doses of growth factor (Wijelath et al., 2006). In order to determine if PDGF and bFGF binding to FN is responsible for the observed adhesion-mediated desensitization, we would need to develop an assay to determine if binding to FN is depleting our stimulation medium of growth factor. If PDGF- and bFGF-depletion via binding to FN is found to occur, depletion of EGF via binding to FN would be tested to determine if a lack of binding explains why no adhesion-mediated desensitization is observed for stimulation via EGF.

Finally, if regulation does not occur at the level of growth factor receptor, we will investigate regulation at the level of Ras. In order to determine if Ras activation is the mechanism responsible for the observed adhesion-mediated desensitization to bFGF/PDGF, but not EGF, the levels of active Ras in PDGF- and bFGF-stimulated adherent cells could be compared to those of suspended cells. Substantial depression of

Ras activation in adherent cells as compared to suspended cells will indicate that desensitization likely occurs at the level of Ras.

4. Adhesion-Dependent Erk Activation Enhancement by EGF Stimulation

4.1 *Background*

A simple coarse-grain mathematical model was developed to focus the search for molecular mechanism(s) driving the magnitude and kinetics of experimentally observed Erk activation profiles. The model demonstrated that while activation, feedback-decoupling deactivation, and one form of direct-deactivation (either constitutive or feedback-mediated) is required for transient signal attainment, a sustained signal is generated provided that feedback-decoupling deactivation never occurs in the presence of any other form of deactivation. Thus, a sustained signaling profile occurs in the presence of activation and (1) no deactivation, (2) a single form of deactivation, or (3) both forms of direct-deactivation.

For transient activation profiles we find that: (1) increasing the magnitude of the activation dimensionless group increases the maximum peak, but that the time at which the signal returns to basal levels is not affected, (2) increasing the decoupling-deactivation dimensionless group depresses peak signal intensity and accelerates the signal's return to basal levels, (3) increasing either constitutive direct deactivation or feedback-mediated direct-deactivation results signal depression without affecting the time at which the signal returns to basal levels, and (4) the presence of constitutive direct

deactivation in place of feedback-mediated deactivation has a greater affect on signaling kinetics.

For sustained activation profiles, we find that the signal reaches a maximum and is maintained at maximum for the entire length of the time course. We also observe that increasing the magnitude of the activation group increases the maximum signal, while increasing the magnitude of any deactivation dimensionless group decreases the maximum signal.

4.2 *Future work*

Focusing first on the mechanism(s) responsible for the presence of adhesion-dependent enhancement of Erk activation signal intensity in cells stimulated via EGF, we note both adherent and suspended cells induce transient activation of Erk. Thus, although according to our model, activation, feedback-decoupling deactivation, and one form of direct deactivation (either constitutive or feedback-mediated) must occur to obtain a transient profile, the important difference in adhesion-dependent Erk activation induced via EGF stimulation is that the peak signal magnitude differs between adherent and suspended cells. Therefore, we look to model predictions to determine which dimensionless group affects the magnitude of signal peak intensity. Our model predicts the enhancement in signal peak magnitude may occur by either an increase in the activation rate constant (κ_{Act}) or an decrease in a deactivation rate constant (κ_{Const}, κ_{Dir}, or $\kappa_{Decoupl}$). Notably, our model demonstrates that variation in the activation rate constant has the greatest affect on signal peak magnitude

In addition, adhesion-mediated signal enhancement is reported for a wide number of signaling proteins including Fak (Guilherme et al., 1998), Ras (DeMali et al., 1999), Raf (Lin et al., 1997), and Mek (Renshaw et al., 1997) activation. Thus, an increase in κ_{Act} is the most likely explanation for the observed phenomena. Note that because the relative importance of activation pathways cannot be determined by the model at this stage of development, all known forms of activation must be considered. In order to focus the mechanistic search, we will begin our investigation of activation pathways by determining the Akt activation profile for adherent and suspended cells. Because Akt is activated by both Ras and growth factor receptors, equivalent Akt activation levels in adherent and suspended cells indicate that regulation of adhesion-induced enhancement of Erk affects the MAPK signaling pathway below the level of Ras activation. In contrast, adhesion-dependent Akt activation suggests regulation occurs at or above the level of Ras activation. Knowledge of whether adhesion-induced enhancement occurs (1) downstream of Ras activation or (2) at or upstream of Ras activation will elucidate which integrin-activation pathways should be more thoroughly investigated.

However, when investigating these activation pathways as possible mechanisms, it is important not to overlook differences in the deactivation dimensionless group(s) in adherent and suspended cells as potential points of regulation for adhesion-dependent Erk activation. Because enhancement of deactivation pathways has never been reported to contribute to adhesion-dependent signal enhancement, possible contributions from feedback-decoupling deactivation and/or direct deactivation will only be investigated if

no enhancement of activation pathway(s) are determined to be of mechanistic importance to adhesion-mediated enhanced of Erk signaling.

5. Growth-Factor Specific Signaling Dynamics of Erk Activation in Adherent Cells

5.1 *Background*

We focus now on the mechanism responsible for growth factor specific Erk signaling dynamics. Our experiments revealed that while adherent cells stimulated by either EGF or PDGF generate transient Erk activation, adherent cells stimulated via bFGF produce sustained Erk activation. According to our model, activation, feedback decoupling, and at least one form of direct deactivation (either constitutive or feedback-mediated) are necessary for the transient activation of Erk observed for adherent cells stimulated via EGF or PDGF. In contrast, our model predicts that either feedback decoupling alone or any form(s) of direct deactivation are necessary for the sustained Erk activation profile observed in cells stimulated via bFGF.

5.2 *Future work*

Recall that feedback-decoupling deactivation is known to occur in cells stimulated via PDGF (Figure III-6, page III-16). As one of our model assumptions is that feedback decoupling is irreversible, this finding gives us the opportunity to validate our model assumptions via assaying temporal Sos hyperphosphorylation profiles in adherent cells stimulated via PDGF. Importantly, if hyperphosphorylated proteins are found to return to

their active unphosphorylated state over the time-course of the experiment, the assumption of irreversible feedback would have to be revised. Current modeling equations would then be modified to include reversibility of feedback-decoupling deactivation and the resulting predictions from the modified model would be used to further guide our search. However, if our assay reveals that decoupling feedback renders proteins permanently inactive, maintaining them in the hyperphosphorylated form throughout the duration of the experiment, our assumption of irreversible feedback decoupling is valid. Current modeling predictions can be used in the continued search for the molecular mechanism responsible for differences in Erk signaling dynamic by different growth factors.

To search for the molecular mechanism responsible for PDGF-mediated stimulation inducing transient Erk activation, while bFGF-mediated stimulation induces a sustained Erk activation, we will investigate if the presence of decoupling-feedback due to PDGF treatment is the point of regulation. We will determine whether feedback-decoupling deactivation occurs via bFGF-mediated stimulation by assaying for protein hyperphosphorylation of Sos, Raf, Mek, and Fak, using techniques described previously. To assay if decoupling deactivation due to adhesion-mediated receptor sequestration or ECM protein sequestration of growth factor ligands are responsible, further investigation and development of assays to ascertain the presence of these forms of decoupling deactivation must occur.

If feedback-decoupling deactivation is not found to occur due to bFGF-stimulation of adherent cells, feedback-decoupling deactivation in cells stimulated via PDGF, and not in cells stimulated via bFGF, would be the mechanism responsible for the observed transient Erk activation resulting from PDGF-stimulation and sustained Erk activation produced by bFGF-stimulation.

In contrast, if feedback-decoupling deactivation is found to occur in cells stimulated by bFGF, the model indicates that direct-deactivation should be investigated as point of regulation in Erk signaling kinetics. The presence of direct deactivation in cells stimulated by PDGF, but not bFGF, would explain the observation that a transient signal is obtained for PDGF-stimulated cells, while a sustained signal for is obtained in bFGF-stimulated cells. The most comprehensive method to test for direct deactivation is to develop a phosphatase assay that uses a kinase inactive form of Erk as a substrate. Phosphatase activity measured by this assay prior to growth factor-stimulation would indicate the presence of constitutive direct-deactivation, while an increase in phosphatase activity in growth factor-stimulated cells would indicate that feedback-mediated direct-deactivation is present. However, as development of such an assay is an intensive process, Western blotting against phosphatases known to act on Erk (such as Mkp1 and Mkp4) is a suitable alternative method to assay for the presence of direct-deactivation. The presence of phosphatases via Western blotting before growth factor stimulation would again indicate the presence of constitutive direct-deactivation, while the upregulation of phosphatases levels after growth factor-stimulation would reveal the presence of feedback-mediated direct deactivation. If Western blotting reveals either the

presence of constitutive or feedback-mediated direct-deactivation, further development of the phosphatase assay will be unnecessary, as direct deactivation would be revealed as the mechanism responsible for PDGF-mediated transient Erk activation and bFGF-mediated sustained Erk activation.

Determination of the mechanism responsible for the dynamic differences in cells stimulated via EGF, compared to those stimulated via bFGF will be identical to the process outlined above for PDGF. The only difference is that the form of feedback-decoupling deactivation that occurs in cells stimulated via EGF must be determined following the methodology outlined previously to determine the source of feedback-decoupling deactivation that occurs in adherent cells stimulated by EGF.

6. Conclusions

Our experimental findings (Chapter 3) have begun to elucidate quantitative aspects of the crosstalk between adhesion and individual growth factors. We have laid out a process for elucidating the precise mechanisms responsible for: 1) adhesion-mediated desensitization of signal activation by selective growth factors, 2) adhesion-dependent Erk activation enhancement by EGF stimulation, and 3) growth factor-specific signaling dynamics of Erk activation in adherent cells. Many of the proposed experiments are guided by model predictions that identify key mechanisms required to elicit specific aspects of Erk signaling dynamics. Pursuing the proposed experiments will yield mechanistic understanding of the quantitatively intricate ways in which cell adhesion influences growth factor signaling. This understanding will offer insights into

how the adhesive microenvironment 'primes' cell behaviors such as survival, apoptosis, proliferation, and migration. Such quantitative insight will enhance our ability to both design synthetic microenvironments for applications such as tissue engineering and regenerative medicine. In addition, quantification will enable improved assessment of the quality and effectiveness of both biomaterials and cancer therapies.

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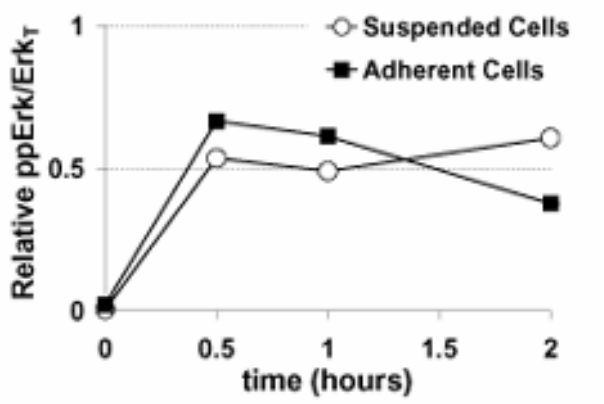
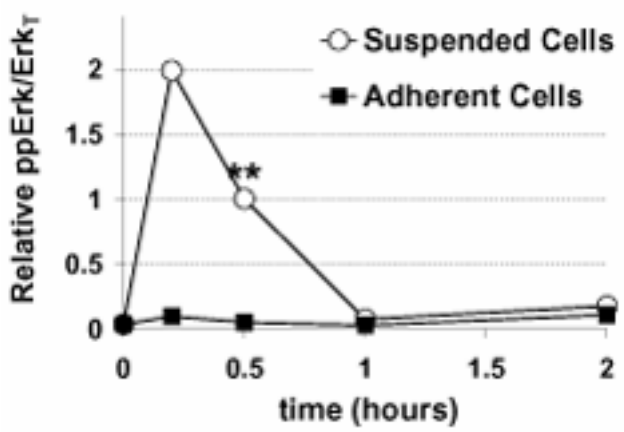
Chapter VI. Appendices



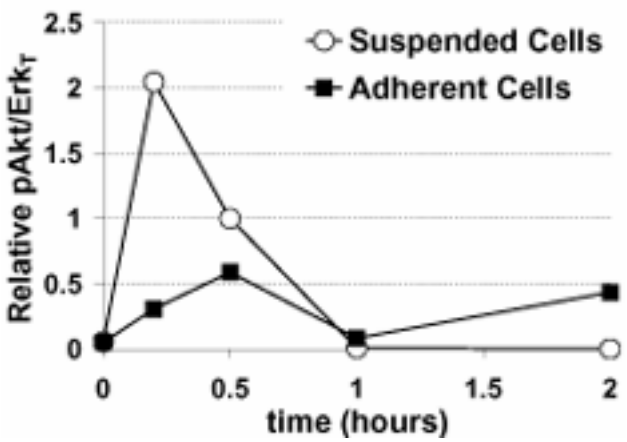
Supplemental Figure VI-1: bFGF does not induce Erk signaling in an adhesion-dependent manner for any portion of the time course.

NIH-3T3 cells were prepared as described in the legend to Figure III-1. Cells that were either maintained in suspension on PH (empty circles) or allowed to adhere on FN (filled squares) for 2.5 hr were stimulated with 1000 pM bFGF for the indicated times prior to lysing. The relative amount of active Erk (ppErk) normalized to the equal-loading control, total Erk (Erk_T), is reported for the different treatment conditions. The graph represents a single experiment.



Supplemental Figure VI-2: At the critical bFGF concentration of 1 pM, suspended cells strongly induce Erk activation, while Erk activation remains near basal in adherent cells.

NIH-3T3 cells were prepared as described in the legend to Figure III-1. After being maintained in suspension by PH (empty circles) or allowed to adhere on FN (filled squares) for 2.5 hr, cells were stimulated with the critical concentration of 1 pM bFGF. The relative amount of active Erk (ppErk) normalized to the equal-loading control, total Erk (Erk_T), is reported for the different treatment conditions. The graph represents one to three independent experiments, with $n > 1$ for the 0 and 30' time point. The double asterisk denotes that 1 pm bFGF-mediated ERK activation in suspended and adherent cells is statistically different ($P < 0.01$).



Supplemental Figure VI-3: At the critical bFGF concentration of 1 pM bFGF, suspended cells also strongly induce Akt activation, while Akt activation remains near basal in adherent cells.

NIH-3T3 cells were prepared and analyzed identically to those in Figure III-1. Total cell lysates were assayed by immunoblot analysis using antibodies specific to total Erk (Erk_T) and phosphorylated Akt (pAkt). The relative amount of active Akt (pAkt) normalized to the equal-loading control, total cellular Erk (Erk_T), is reported for the different treatment conditions. The graph represents a single experiment.