Quantification of Growth Factor Signaling and Pathway Crosstalk by Live-Cell Imaging

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Abstract

Peptide growth factors stimulate cellular responses through activation of their trans-membrane receptors. Multiple intracellular signaling cascades are engaged following growth factor – receptor binding, leading to short- and long-term biological effects. Each receptor-activated signaling pathway does not act in isolation, but rather interacts at different levels with other pathways to shape signaling networks that are distinctive for each growth factor. To gain insights into the specifics of growth factor-regulated interactions among different signaling cascades, we developed a HeLa cell line stably expressing fluorescent live-cell imaging reporters that are readouts for two major growth factor-stimulated pathways, Ras – Raf – Mek – Erk and PI3-kinase – Akt. Incubation of cells with EGF resulted in rapid, robust, and sustained Erk signaling but shorter-term activation of Akt. In contrast, HGF induced sustained Akt signaling, but weak and short-lived Erk activity, and IGF-I stimulated strong long-term Akt responses, but negligible Erk signaling. To address potential interactions between signaling pathways, we employed specific small molecule inhibitors. In cells incubated with EGF or PDGF-AA, Raf activation and the subsequent stimulation of Erk reduced Akt signaling, while Mek inhibition, which blocked Erk activation, enhanced Akt, and turned transient effects into sustained responses. Our results reveal that individual growth factors initiate signaling cascades that vary markedly in strength and duration, and demonstrate in living cells the dramatic effects of crosstalk from Raf and Mek to PI3-kinase and Akt. Our data further indicate how specific growth factors can encode distinct cellular behaviors by promoting complex interactions among signaling pathways.

Keywords: growth factors, cell signaling, signal transduction, signaling dynamics, Akt/PKB, Raf, Mek, Erk, live-cell imaging
Introduction

Peptide growth factors mediate the responses of cells to environmental stimuli by regulating the activity of intracellular signaling pathways (11). Growth factor actions begin with receptor binding, which triggers intracellular protein kinase enzymatic activity, leading to the stimulation of multiple downstream signaling networks that typically function through a series of phosphorylation cascades (11, 26). Despite advances in cell biology and biochemistry that have identified and characterized receptors and downstream pathways in great detail, our knowledge of signaling dynamics and how growth factor-initiated inputs are encoded into distinctive cellular outputs remains limited (25, 37). Studying the responses of single cells in a population has been particularly challenging because most experimental methods exhibit inadequate sensitivity and temporal resolution. Moreover, these approaches are unable to account for potential interconnections, or feedback and feed-forward loops that might modify the duration and magnitude of individual signaling pathways (3, 37, 55, 57).

The Ras – Raf – Mek – Erk cascade has long served as a canonical example for studying signaling outcomes downstream of tyrosine kinase receptors. In seminal work, it was found that different growth factors induced distinct fates in PC12 cells in a manner related to the duration of Erk activity (29). More recently, progress has been made in understanding mechanisms underlying Erk signaling dynamics. Optogenetic methods have been developed that allow manipulation of the magnitude and duration of Erk activity (60), and fluorescent reporters have been generated that enable the tracking of responses in individual cells (3, 9, 38, 45). Collectively, both older and newer studies have shown that Erk can produce pulsatile or sustained downstream effects, depending on the cell type and the pattern of growth factor exposure (3, 9, 45), and have revealed an important role for negative feedback in modulating signaling outcomes (10, 24, 47, 55, 64).
The PI3-kinase – Akt signaling pathway is also stimulated by growth factors via their tyrosine kinase receptors (28). Active Akt can directly phosphorylate several categories of substrates, including mediators of immediate changes in intermediary metabolism, and cell shape and movement, and can promote long-term effects on cell viability, proliferation, or differentiation (18, 28, 61). Recently, using a live-cell imaging approach with a fluorescent translocation sensor, we characterized Akt signaling responses induced by different growth factors in a fibroblast cell line. Our studies showed that signaling dynamics and outcomes were growth factor-specific (15). IGF-I1 and insulin produced sustained concentration-dependent effects, whereas EGF caused more transient responses. PDGF-BB and PDGF-AA mediated transient effects at lower growth factor exposures, and produced sustained responses at higher concentrations. Moreover, in results analogous to earlier observations on Erk pathway-mediated changes in cell fate, we found that the duration of Akt signaling was related to the ability of individual growth factors to promote cell cycle progression (16).

Our initial observations focusing on a single signaling mediator illustrated the potentially critical roles played by individual growth factor receptors in determining signaling outcomes (15, 16). Each receptor, with its distinct pattern of phospho-tyrosine-specific binding domains for signaling intermediates, also may shape the extent of activation of different downstream signaling cascades (35, 41, 62). As a consequence, receptors may be key factors leading to biased stimulation of one pathway versus another and thus help determine how different growth factors induce distinct biological outcomes (2, 32, 41). This interactive process of crosstalk between pathways has many potential ramifications. For instance, in some cells, Ras can stimulate PI3-kinase, thus leading to Akt activity (2, 22, 32, 43, 63). As a result, a growth factor receptor that directly activates the Ras – Raf – Mek – Erk pathway also may indirectly promote Akt signaling.
In this report, we have evaluated in real time both the Ras – Raf – Mek – Erk and PI3-kinase – Akt signaling pathways in the same cells using fluorescent translocation sensors. Our results show that different growth factors not only stimulate distinct signaling dynamics, but also produce biased activation of one pathway over another. Moreover, we find that crosstalk from Ras – Raf – Mek – Erk to PI3-kinase – Akt has pronounced modulatory effects on Akt signaling activity. Our observations thus provide a quantitative insight into how different growth factors control cellular behaviors, and how individual signaling pathways influence each other.
Materials and Methods

Materials. Cell culture media, including Dulbecco’s modified Eagle’s medium (DMEM), phosphate-buffered saline (PBS), and FluoroBrite imaging medium, and trypsin/EDTA solution, were purchased from Gibco-Life Technologies (Carlsbad, CA). FBS was from Hyclone (Logan, UT). Cells were grown and imaged on 6-well Bio-One tissue culture plates (Greiner, Monroe, NC). Restriction enzymes, buffers, ligases, and polymerases were obtained from BD Biosciences-Clontech (Palo Alto, CA) and Roche Applied Sciences (Indianapolis, IN). Primary antibodies were purchased from Cell Signaling (Beverly, MA): anti-phospho-PRAS40 (catalog #2997), anti-PRAS40Thr246 (#2691), anti-phospho-AktThr308 (#2965), anti-phospho-AktSer473 (#9271), anti-Akt (# 2691), anti-RskThr359 (# 8753), anti-Rsk (#9355), anti-phospho-Erk1/2Thr202/Tyr204 (#4370), and anti-Erk (#4695). Secondary antibodies included IR800-conjugated goat anti-rabbit IgG (Rockland, Gilbertsville, PA), and goat anti-rabbit-IgG conjugated to Alexa Fluor 680 (Invitrogen, Carlsbad, CA). The IGF-I analogue, R3-IGF-I, was purchased from GroPep (Adelaide, Australia), recombinant human HGF and recombinant human TGF-α were from ProSpec (Rehovot, Israel), recombinant human insulin was from Tocris Bioscience (Bristol, United Kingdom), mouse EGF was from Gibco-Life Technologies, and recombinant human PDGF-AA was from Thermo-Fisher Scientific (Rockford, IL). Growth factors were dissolved in 10 mM HCl with 1 mg/ml bovine serum albumin, stored in aliquots at -80°C, and diluted into FluoroBrite imaging medium immediately prior to use. Small molecule inhibitors were purchased as follows: PI103 and UO126 (Tocris), trametinib (LC Laboratories, Woburn, MA), PLX-4720 and MK-2206 (Selleck, Houston, TX). All inhibitors were solubilized in DMSO and diluted into FluoroBrite imaging medium just prior to use. The fluorescent signaling protein array (Pathscan Antibody Array Kit #9700S) was purchased from Cell Signaling. Other reagents and chemicals were obtained from commercial suppliers.
Production of recombinant lentiviruses and stable cell lines. The FoxO1-clover recombinant lentivirus has been described (14). To construct the mKate2-Erk2 lentiviral plasmid, tagBFP was replaced with mKate2 in plasmid pHRSFFVp BFP-Erk2 (Addgene, Cambridge, MA #50848); the DNA fragment containing mKate2-Erk2 was then ligated into the pWPXL lentiviral plasmid in place of EGFP (Addgene, #12257). Lentiviruses were prepared as outlined previously (13, 59). HeLa cells [ATCC #CCL-2] were co-transduced with concentrated mKate2-Erk2 and FoxO1-clover lentiviruses in the presence of polybrene (6 μg/ml). Seven days later, cells were sorted by fluorescence intensity (green, excitation 488 nm, emission 530/40 nm) and (red, excitation 561 nm, emission 615/24 nm) using a Becton-Dickinson Influx cell sorter at the Oregon Health & Science University Flow Cytometry Core Facility. Cell populations were obtained that expressed both FoxO1-clover and mKate2-Erk2. The C3H10T1/2 stable cell line expressing FoxO1-clover was previously described (14).

Live cell imaging. Cell imaging was performed using an EVOS FL Auto microscope with a stage top incubator maintained at 37°C and 5% CO2. Images were collected at 100X magnification using a 10X Fluorite objective (numerical aperture: 0.3), with GFP (green: excitation peak, 472/22 nm; emission peak, 510/42 nm) and Texas Red LED light cubes (red: excitation peak 585/29 nm; emission peak, 624/40 nm). Data were analyzed with the NIH ImageJ processing package (Fiji, NIH, Bethesda, MD) using the Subtract Background plug-in to remove background fluorescence, the Stack Reg plug-in (rigid registration) to register images, and the Gaussian Blur plug-in (2-pixels) to reduce pixel-pixel noise (48). To quantify the subcellular translocation of reporter proteins in HeLa cells, nuclei were manually located and labeled using the ROI Manager plug-in following a 2-hr incubation in SFM. This was defined as time 0. HeLa cells underwent minimal migration over a 90 min imaging period, such that nuclear x/y coordinates at time 0 could be propagated across the entire image stack using the Multi Measure tool. The location of each nucleus from the green channel image (FoxO1-clover) was used to quantify nuclear intensity of the red channel image (mKate2-Erk2). Nuclear
intensities for FoxO1-clover and mKate2-Erk2 in each cell were normalized to the value recorded at time 0, and then scaled based on the average maximal reporter translocation after incubation of cells with EGF (SFM = 0%, peak EGF = 100%). To measure the subcellular distribution of FoxO1-clover in C3H10T1/2 cells the nuclei of individual cells were manually tracked across frames using the mTrackJ plug-in (31). Cells that died, divided, or migrated out of frame were excluded from analysis.

**Imaging protocols.** HeLa cells were grown in DMEM containing 10% FBS for 48 hr to allow full cell attachment. After two washes with DMEM, cells were incubated in serum-free Fluorobrite imaging medium for 2 hr. Growth factors and/or inhibitors were then added and images were collected every 2.5 min for 90 min. Growth factors included R3-IGF-I [0 to 250 pM], EGF [0 to 2.1 nM], HGF [0 to 1.7 nM], insulin [1 nM], and TGF-α [1.67 nM]. For inhibitor studies, HeLa cells were incubated in SFM for 2 hr followed by addition of growth factor (EGF [2.1 nM], R3-IGF-I [250 pM], or HGF [1.72 nM]), with or without PI103 [500 nM], UO126 [10 μM], trametinib [500 nM], or MK-2206 [1 μM]. C3H10T1/2 cells were incubated in SFM for 90 min, followed by the addition of growth factors (EGF [2.1 nM] or PDGF-AA [1.4 nM]) with or without PI103 [500 nM], UO126 [10 μM], or PLX-4720 [10 μM]. Images from C3H10T1/2 cells were collected every 2 min for 90 min. For all imaging studies a minimum of 3 independent experiments were performed.

**Protein extraction and immunoblotting.** Whole cell protein lysates were collected after washing cells twice with cold PBS followed by the addition of extraction buffer containing protease and phosphatase inhibitors. Protein aliquots (12.5 μg/lane) were separated by SDS PAGE (12% separating gels), followed by transfer to Immobilon-FL membranes, blocking with 50% AquaBlock solution, and sequential incubation of membranes with primary and secondary antibodies, as described (34). Primary antibodies were added at 1:1000 dilutions for 16 hr at 4°C, and secondary antibodies at 1:5000 dilution for 90 min at 20°C. Images were captured using the
LiCoR Odyssey and version 3.0 analysis software (Lincoln, NE). For analysis by protein array, whole cell protein lysates were collected from HeLa cells after addition of the provided cell lysis buffer (Cell Signaling: Pathscan Antibody Array Kit) supplemented with protease inhibitors. Protein aliquots (75 μg/well) from cells incubated with SFM or EGF [2.1 nM] for 15 min ± PI103 [500 nM] and/or UO126 [10 μM] were added to each slide well and incubated at 4°C for 16 hr. Slides were washed 4 times with the provided wash buffer and then incubated for 1 hr with the detection antibody cocktail at 20°C. Following 4 additional washes at 20°C, DyLight 680®-linked Streptavidin from the kit was added to each well and incubated for 30 min at 20°C. After 4 final washes, slides were dried, and visualized with the LiCoR Odyssey.
Results

Growth factor signaling in living cells. Growth factors typically regulate a broad range of cellular responses by engaging multiple intracellular signaling pathways (11). Although each growth factor binds to its unique receptor, many downstream cascades are shared, leading to the question of how individual growth factors can cause distinct behavioral responses. We recently developed a fluorescent reporter protein designed to measure Akt activity at the single cell level (14, 15). The reporter, which is composed of a modified portion of the well-characterized Akt substrate, FoxO1 (6, 39, 40, 66), fused at its COOH terminus to the green fluorescent protein, clover (23), underwent movement from the nucleus to the cytoplasm when phosphorylated by Akt (14, 15) (Fig. 1A, right panel). We found that different peptide growth factors elicited distinctive dynamic patterns of reporter translocation in living cells that matched their ability to stimulate Akt activity, and that signaling strength and duration varied substantially among individual cells in a population (14, 15).

In order to evaluate a broader template of growth factor-mediated signaling, we generated recombinant HeLa cell lines incorporating two live-cell imaging sensors, FoxO1-clover and mKate2-Erk2 (Fig. 1A, left panel). The latter fluorescent reporter reads the kinase activity of Mek 1 and 2, upstream stimulators of Erk 1 and 2, and of phosphatases that dephosphorylate the corresponding sites (51). Upon phosphorylation of Erk by Mek, the reporter accumulates in the nucleus (Fig. 1A, left panel). Incubation of these HeLa cells with a high dose of EGF [2.1 nM] demonstrated nearly coordinate activation of both Akt and Mek. In the population, translocation of mKate2-Erk2 from the cytoplasm to the nucleus was maximal within 5 min of EGF addition, declined to a plateau that was ~70% of maximal by 15 min, and was maintained at this level of activity for at least 90 min, although this varied among individual cells (Fig. 1B, C, movie 1 in supplemental data). In the same cell population, FoxO1-clover maximally translocated from the nucleus to the cytoplasm within 7.5 – 10 min after EGF addition. Cytoplasmic localization then
declined at a steady rate to ~30% of maximal at 90 min, despite the continuing presence of
growth factor (Fig. 1B, D, movie 1 in supplemental data), although both maximum cytoplasmic
accumulation and the rate of return of the reporter to the nucleus varied among single cells (Fig.
1D). Thus, treatment of HeLa cells with high concentrations of EGF stimulates robust Mek
signaling responses, but less sustained Akt activity, and tracking the subcellular distribution of
translocation-based sensors by live-cell imaging provides a dynamic picture of signaling with
high temporal resolution. Maryu et al have recently reported on a similar dual-reporter model in
which they also have studied the effects of EGF (30).

To determine if reporter behavior in living cells tracked with more traditional measures of
growth factor activated signaling, we performed serial immunoblotting of lysates from HeLa
cells exposed to the same concentrations of EGF for variable times. Erk phosphorylation was
minimal in cells incubated in SFM, and rose to a peak within 5 min of EGF addition. This level
of Erk phosphorylation declined by 30 min, and was maintained at the lower value for at least 90
min after growth factor addition (Fig. 1E). A relatively similar pattern of phosphorylation was
observed for the Erk substrates, Rsk1 and 2 (Fig. 1E). Akt phosphorylation also peaked within 5
min of cell exposure to EGF, and then rapidly fell, such that it was only marginally more than
baseline by 90 min (Fig. 1E). The direct Akt substrate, PRAS40, showed a similar
phosphorylation pattern to Akt (Fig. 1E). Thus, live-cell imaging using translocation sensors that
reflect Mek and Akt kinase activity reveals signaling dynamics that are comparable to those seen
by immunoblotting.

Variable signaling responses in HeLa cells to different growth factors. To test the effects of
different growth factors on signaling dynamics, we exposed HeLa cells to varying concentrations
of EGF, HGF, or IGF-I for up to 90 min (Figs. 2 and 3). Addition of EGF to cells pre-incubated
with SFM caused a rapid, dose-dependent peak in nuclear translocation of the mKate2-Erk2
reporter molecule. At the population level, half-maximal accumulation of the reporter in the
nucleus was detected by 3 – 5 min after EGF addition at all growth factor concentrations tested, with maximal values being attained by ~7 min. Signal intensity then rapidly waned by 15 min to a steady state value that was sustained for up to 90 min at a level ranging from ~0% to ~50% of peak value depending on the EGF concentration (Fig. 2A, left panel).

The signaling response of FoxO1-clover to EGF varied from what was seen with mKate2-Erk2. Half maximal nuclear to cytoplasmic translocation occurred within ~5 min of growth factor addition, and maximal activity was attained by ~10 min. This was followed by a nearly linear fall over the subsequent 20 to 80 min to a baseline value that was maintained for the duration of each 90 min experiment, with the kinetics and levels of activity depending on EGF concentration (Fig. 2B, left panel). For both pathways, the lowest EGF concentration tested [11 pM] gave a peak response that was ~50% of the highest dose [1.1 nM] (Fig. 2A, B, left panels). Thus, at the population level EGF caused dose-dependent stimulation of Ras – Raf – Mek – Erk and PI3-kinase – Akt signaling in HeLa cells.

HGF promoted different signaling dynamics than did EGF. Translocation of the mKate2-Erk2 reporter was transient, peaking within ~8 min and declining to basal values by ~20 min, with peak magnitude varying with HGF concentration (Fig. 2A, right panel). At the highest levels of HGF exposure [~1.7 nM], the maximal extent of nuclear localization of mKate2-Erk2 was only half of what was observed in response to EGF (compare left and right panels in Fig. 2A). By contrast, nuclear to cytoplasmic translocation of FoxO1-clover in cells incubated with HGF was sustained. Maximal cytoplasmic accumulation of the reporter was observed within ~10 – 15 min after growth factor addition, and reached ~80% of the peak values seen after EGF treatment (Fig. 2B, right panel). Unlike EGF, after HGF exposure FoxO1-clover was fully maintained in the cytoplasm in the population for the entire 90 min experimental period (compare left and right panels in Fig. 2B).
At the level of individual cells, the effects of EGF and HGF were both heterogeneous. At intermediate concentrations of each growth factor (EGF [110 pM] and HGF [572 pM]), there was significant variability in peak responses, and in the duration of signaling (Fig. 2C). For example, Mek activity after HGF ranged from transient to sustained, and maximal FoxO1-clover translocation varied by a factor of two (Fig. 2C, right panel). Thus, average population responses may mask substantial individual heterogeneity in growth factor-stimulated pathway activity.

IGF-I-activated signaling differed from the responses to EGF or HGF. In the population as a whole, there was minimal subcellular redistribution of mKate2-Erk2 after IGF-I treatment, even at the highest growth factor concentrations [250 pM] (Fig. 3A, left panel). By contrast, and as shown previously in other cell types (15, 16), IGF-I promoted rapid, robust, dose-dependent, and sustained Akt signaling, as reflected by maximal translocation of FoxO1-clover by ~12 - 18 min that was maintained for the entire 90 min experimental time course (Fig. 3B). Peak values of reporter translocation in response to IGF-I were indistinguishable from those seen with EGF, but were far more sustained (compare Fig. 3B with 2B, left panel). As was seen for EGF and HGF, individual cellular responses to IGF-I [50 pM] were highly variable, with mKate2-Erk2 cytoplasmic to nuclear translocation ranging from minimal to values as high as seen with EGF (compare Fig. 3C with 2C, left panel), and the subcellular redistribution of FoxO1-clover varying over a two-fold range, as noted with HGF (compare Fig. 3C with 2C, right panel).

Additional experiments showed that overall population signaling responses to TGF-α were nearly identical to those seen with EGF, and that responses to insulin mimicked the pattern seen with IGF-I (Fig. 3D). This is not surprising, since TGF-α uses the same receptors as EGF (49), and the insulin and IGF-I receptors employ the same intracellular adaptor molecules to couple with downstream signaling modules (54). Thus, the signaling dynamics that we observed appear to be largely dependent on the specific receptor tyrosine kinases that are activated.
A plot of the maximal responses of mKate2-Erk2 and FoxO1-clover reporters to different growth factors revealed a wide range of signaling patterns in individual cells. Erk and Akt activity were minimal in the vast majority of HeLa cells incubated in SFM (Fig. 3F, black dots). By contrast, both insulin and IGF-I led to substantial stimulation of Akt and the consequent nuclear to cytoplasmic translocation of FoxO1-clover in nearly all cells, with the IGF-I response being on average twice that of insulin (Fig. 3F, green vs. orange dots). In these same cells Erk activity was minimal, and was similar to what was seen in SFM (Fig. 3F, compare green or orange with black dots). In contrast, signaling by HGF, TGF-α, or EGF led to coordinate activation of both pathways, with the maximal effects of EGF and TGF-α being approximately twice the magnitude of HGF, although there was substantial overlap in single cells (Fig. 3F, compare blue and red dots). Furthermore, the single-cell peak effects of EGF, TGF-α, and HGF fell along a diagonal, indicating that in cells treated with these growth factors, high levels of FoxO1-clover translocation tended to be accompanied by high Erk activity. Taken together, the results reveal cell-to-cell heterogeneity in growth factor signaling activity in the population, while also demonstrating correlations of pathway-specific signaling responses in individual cells.

**Potential signaling crosstalk and convergence mechanisms.** The Ras – Raf – Mek – Erk and PI3-kinase – Akt signaling pathways have been reported to exhibit crosstalk at several levels and to show convergence onto similar targets in different cell types and contexts (32). Here we have used small molecule inhibitors to assess the possible interdependence of these cascades. We find that addition of the dual PI3K and mTor inhibitor, PI103, or the Akt inhibitor, MK2206, had no effect on EGF-stimulated Mek activity, but reduced nuclear to cytoplasmic translocation of FoxO1-clover by nearly 50% and 25%, respectively (Fig. 4A). Similarly, translocation into the nucleus of mKate2-Erk2 after HGF treatment was not altered by PI103, but the response of FoxO1-clover was diminished by ~50% (Fig 4B). In contrast, the same concentrations of PI103 or MK2206 that only partially prevented EGF- or HGF-mediated nuclear to cytoplasmic
translocation of FoxO1-clover, were fully effective in blocking the effects of IGF-I in HeLa cells (Fig. 4C). PI103 also completely inhibited the actions of EGF on nuclear to cytoplasmic translocation of FoxO1-clover in C3H10T1/2 cells (Supporting Fig. 1). Taken together, these results suggest that EGF- and HGF-stimulated signaling may regulate FoxO1-clover differently than IGF-I in HeLa cells, most likely secondary to strong Erk actions.

To test this idea of alternative modulation of FoxO1, HeLa cells were incubated with EGF along with PI103 and/or Mek inhibitors, UO126 or trametinib. As expected, each Mek inhibitor completely blocked EGF-stimulated cytoplasmic to nuclear movement of the direct Mek substrate, mKate2-Erk2 (Figs. 5A and B, left panels, movie 2 in supplemental data). Surprisingly, each compound also enhanced the duration of translocation of FoxO1-clover into the cytoplasm (Figs. 5A and B, right panels, movie 2 in supplemental data). In contrast, these small molecules amplified the effects of PI103, leading to nearly complete maintenance of FoxO1-clover in the nucleus after EGF (Figs. 5A and B, right panels). Our overall interpretation of these results is that specific loss of Mek-stimulated Erk activity reduces negative feedback on Ras, which leads to sustained stimulation of PI3-kinase, thus enhancing Akt activity. In contrast, when all paths to Erk and to PI3-kinase and Akt activation are blocked by a combination of Mek and PI3-kinase inhibitors, then the FoxO1-clover sensor remains un-phosphorylated and nuclear.

As a follow up to these studies, we measured the effects of UO126 on EGF-stimulated phosphorylation of signaling intermediates using a protein array. Incubation of HeLa cells with EGF for 15 min led to increased phosphorylation of Mek and Akt substrates, as well as enhanced phosphorylation of downstream effector molecules. UO126 blocked EGF-mediated phosphorylation of Erk and Rsk1, and also inhibited phosphorylation of p70 S6 kinase, and S6, but had a stimulatory effect on phosphorylation of the direct Akt substrate, PRAS40 (Fig. 5C). In contrast, PI103 did not alter EGF-stimulated Erk or Rsk phosphorylation, but impaired EGF-mediated phosphorylation of PRAS40 and S6 (Fig. 5C). When used together, the two inhibitors...
(PI103 and UO126) reduced PRAS40 and S6 phosphorylation below baseline levels. Thus, in HeLa cells, it appears that inhibition of Mek activity increases EGF-activated PI3-kinase - Akt signaling, and that dual blockade of both pathways reduces putative ‘Akt’ and ‘Erk’ substrates very effectively.

Inhibitory crosstalk from Erk to Akt. To broaden the observations made in Figs. 4 and 5, we examined the effects of small molecule inhibitors that act at different points upstream of Erk. The Ras – Raf – Mek – Erk pathway contains several redundancies and negative feedback loops. For instance, phosphorylation by Erk can reduce Ras activity, resulting in diminished downstream signaling (24, 51, 64). Thus, Mek inhibitors not only directly block Erk activation, but also alter upstream pathway components (24).

The small molecule, PLX-4720, can inhibit a mutant constitutively active B-Raf that contains a valine to glutamic acid substitution at amino acid 600, but paradoxically stimulates wild type Raf1 (36). As a consequence, this drug has been found to increase Erk activity. To assess the effects of PLX-4720 on EGF-mediated signaling, C3H10T1/2 cells, which do not show a strong effect of Erk-mediated signaling on FoxO1-clover translocation (Fig. 6A), were incubated in the presence or absence of PLX-4720 or the Mek inhibitor, UO126, and the subcellular distribution of FoxO1-clover was monitored. In these cells, EGF stimulated transient nuclear to cytoplasmic translocation of FoxO1-clover, and this response was potentiated in duration from less than 45 to greater than 90 minutes by UO126 (Fig. 6A). In contrast, reporter translocation was dampened by 20% in magnitude and reduced in duration by PLX-4720 (Fig. 6A). Analogous results were seen after PDGF-AA treatment (Fig. 7A).

Immunoblotting studies revealed similar effects after incubation of cells with EGF for 15 min in the presence or absence of UO126 or PLX-4720. EGF-mediated Akt phosphorylation was
increased by UO126 and reduced by PLX-4720, while Erk phosphorylation was completely
blocked by UO126 and enhanced by PLX-4720 (Fig. 6B).

One key advantage of live-cell imaging with translocation sensors is that all cells in a population
may be analyzed individually, rather than assessed as a group average (37). Examination of the
effects of EGF on the subcellular distribution of FoxO1-clover revealed heterogeneity in both the
magnitude and duration of responses in single fibroblasts. Co-incubation with UO126 enhanced
the duration of peak signaling after EGF in the majority of cells (compare Fig. 6C and D). By
contrast, addition of PLX-4720 reduced the time course of FoxO1-clover nuclear to cytoplasmic
translocation (Fig. 6E). However, in both populations, marked individual cell variability
remained. Analogous results were seen after treatment of cells with PDGF-AA (Fig. 7B-D).

Thus, these results show that crosstalk occurs between the Ras – Raf – Mek – Erk and PI3-kinase
– Akt cascades in response to EGF and PDGF-AA, demonstrating in C3H10T1/2 cells as well as
in HeLa cells, that Mek inhibition potentiates Akt activity (Fig. 7E).
Discussion

Growth factors bind to distinct trans-membrane receptors and stimulate multiple intracellular signaling cascades that influence cellular behavior by modifying intermediary metabolism, altering cell shape and movement, changing gene expression, and regulating the rate and extent of cell proliferation or differentiation (26). Each signaling pathway typically consists of a series of interacting proteins that previously were thought to comprise a simple linear pattern, and to function in isolation. It is now clear from many cellular and biochemical studies that individual signaling cascades contain multiple feedback and feed forward loops, and have extensive interconnections with other pathways (25, 32, 63). Here, we have used fluorescent live-cell imaging sensors to simultaneously track the effects of different growth factors on two major signaling cascades, the Raf – Ras – Mek – Erk and PI3-kinase – Akt pathways. Our results reveal that individual growth factors initiate signaling responses that vary markedly in strength and duration, and demonstrate in living cells the dramatic effects of crosstalk from Raf and Mek to PI3-kinase and Akt, as well as the potential convergence of Erk-mediated signaling onto Akt signaling substrates like FoxO1. Our observations illustrate the dynamics of growth factor-initiated signaling cascades in real time, and provide insights into how individual growth factors can encode distinct cellular behaviors through the promotion of complex interactions between co-regulated pathways.

Discordant signaling pathway dynamics are controlled by crosstalk. Although it has been shown that different growth factors acting through tyrosine kinase receptors stimulate analogous intracellular signaling pathways, overall signaling strength and duration varies considerably among individual growth factors and cell types (5, 7, 14, 20, 35, 63). Here, we find dramatic differences in signaling dynamics in HeLa cells among three different growth factors. We show that EGF stimulated rapid activity of Mek, consisting of a brief peak, followed by a sustained plateau. Akt signaling in response to EGF also reached a rapid peak, but then fell at a relatively
constant rate in the majority of cells studied. HGF promoted similar kinetics of Mek-mediated signaling to Erk, but with a magnitude that was less than half that of EGF. In contrast, sustained stimulation of Akt activity by HGF resembled the pattern seen with IGF-I, which only minimally activated Mek in most cells (Figs. 2 and 3). Furthermore, pairing the peak mKate2-Erk2 and FoxO1-clover responses revealed cell-to-cell heterogeneity across the population, but a correlation within individual cells (Fig. 3F).

Biased signaling responses favoring one cascade over another presumably reflect the nature of the respective growth factor receptors, downstream adaptors, feedback mechanisms, and/or crosstalk pathways, all of which potentially vary among EGF, HGF, and IGF-I (5, 25, 26, 32). EGF binds primarily to EGFR (HER1), and through hetero-dimerization also can activate HER2, HER3, and HER4 (8, 42). HGF binds to the extracellular surface of a homo-dimeric receptor composed of identical subunits of the tyrosine kinase Met proto-oncogene, which then through intracellular phospho-tyrosine binding sites interacts with a range of signaling modules (12, 46). In contrast, IGF-I binds to the hetero-tetrameric IGF-I receptor, which primarily engages intracellular signaling pathways through adaptor molecules such as IRS1 and IRS2 (54).

Small molecule pathway modifiers are useful reagents in live-cell imaging-based signaling experiments, as they allow rapid and fairly specific interference at defined steps in a cascade. Blocking Mek activity with either of two different chemical inhibitors led to sustained EGF-stimulated Akt activity that resembled the effects of IGF-I, as under these conditions Erk was minimally activated (compare Fig. 4A, 4B, or 5A with Fig. 3A), thus illustrating that crosstalk at different levels of the Raf – Ras – Mek – Erk pathway may normally impair PI3-kinase – Akt signaling. As similar results were observed after EGF exposure in two different cultured cell types (Figs. 4 - 6) and also in cells treated with PDGF-AA (Fig. 7), our data demonstrate that this type of inhibitory crosstalk may be generalizable, and is consistent with previous work using more standard approaches (22, 43, 56). Published studies have found that inhibiting Erk
signaling at Mek leads to a block in negative upstream pathway feedback from Erk to Ras (24, 51). As a consequence Ras activity is not down regulated, and can continue to directly stimulate PI3-kinase and thus Akt (see schematic in Fig. 7E). Although through this mechanism Erk activity can modulate Akt signaling, this type of interaction only seems to occur when a prior signal also stimulates the PI3-kinase – Akt cascade, since direct induction of Ras using optogenetic methods did not promote PI3-kinase or Akt activity (60).

Consistent with observations using Mek inhibitors, activation of Raf1 by the small molecule, PLX-4720, caused a more rapid decline in Akt signaling in cells incubated with either EGF or PDGF-AA compared with controls (Figs. 6A and 7A). This result provides additional validation that activation of the Raf – Ras – Mek – Erk signaling cascade negatively impacts PI3-kinase – Akt activity. Of note, however, there were significant differences in overall signaling responsiveness to EGF compared with PDGF-AA. In the absence of chemical inhibitors, PDGF-AA caused more sustained Akt activity, with nuclear to cytoplasmic translocation of the FoxO1-clover reporter lasting for over 90 min versus 45 min for EGF (compare Figs. 7A and 6A). In contrast, effects of Raf1 activation by PLX-4720 were more dramatic in cells incubated with EGF, where Akt activity was inhibited far below basal levels, while signaling only returned to baseline in cells treated with PDGF-AA (compare Figs. 6A and 7A). These results probably reflect intrinsically stronger stimulation of the Raf – Ras – Mek – Erk pathway by EGF, and of the PI3-kinase – Akt cascade by PDGF-AA in C3H10T1/2 cells, and thus demonstrate biased signaling, in which one pathway is preferentially activated over another by a given growth factor.

In other cell types, crosstalk from the PI3-kinase – Akt pathway to the Raf – Ras – Mek – Erk cascade via inhibitory phosphorylation of Raf1 by Akt also has been identified (17, 33, 67). In our studies, we only found a small reduction in EGF- or HGF-treated cells in cytoplasmic to nuclear translocation of the mKate2-Erk2 reporter by either the Akt inhibitor, MK2206, or the dual PI3-kinase and mTorc inhibitor, PI103 (Fig. 4A, B). Thus, in HeLa cells, we observed little
reciprocal crosstalk from PI3-kinase or Akt to control Mek activity, potentially illustrating cell-
type specificity.

In addition to crosstalk, distinct signaling pathways may converge on the same downstream
proteins (32). For example, the Akt and Rsk kinases recognize similar amino acid sequences,
and thus could phosphorylate some of the same substrates, leading to similar biological effects (4,
28, 52). There is also the potential for convergence of two pathways through phosphorylation at
different sites in the same target protein (65). These mechanisms could explain why EGF- or
HGF-stimulated nuclear to cytoplasmic translocation of FoxO1-clover in HeLa cells was not
blocked completely either by the Akt inhibitor, MK2206, or by the PI3-kinase-mTorc inhibitor,
PI103, but was fully impaired by combined treatment including a Mek inhibitor (Fig. 5A, B). [ 5

Limitations and implications of dual-reporter live-cell imaging for cell signaling. One issue
that could limit the impact of our observations is the potential contribution of Erk over-
expression on overall cell signaling dynamics, as our reporter molecule contains a functional
Erk2. However, unlike previous studies, we did not detect nuclear localization of the reporter
protein in the absence of growth factor activity (1, 27). Moreover, it is clear that our live-cell
imaging sensors do not occupy parallel positions in the PI3-kinase – Akt and Raf – Ras – Mek –
Erk signaling pathways, respectively. FoxO1 is one of a multitude of direct substrates of Akt
(28), and thus the FoxO1-clover fusion protein measures the activity of a signaling enzyme with
many intracellular targets. In contrast, Erk1 and Erk2 are essentially the only specific substrates
for Mek1 and Mek2 (51), and the subcellular translocation of mKate2-Erk2 thus does not give
information equivalent to the redistribution of FoxO1-clover on overall signaling dynamics or on
the comprehensive nature of crosstalk. Future live-cell imaging studies could use an Erk
substrate rather than Erk2 to better address these concerns, which would enable a more precise
comparison of overall Akt and Erk signaling activity in the same cells.
From a broader perspective, we have attempted to use live-cell imaging to study the dynamics of two major pathways, yet we acknowledge that our results are limited in the context of overall cellular signaling, since additional growth factor-activated cascades also exert significant effects on cellular behaviors (26). For example, readouts from other MAP kinase pathways, Stats, Src kinases, and protein kinase C cascades, as well as micro-RNAs, also play important roles in the short- and longer-term responses to growth factors, and to cytokines and hormones (19, 21, 44, 50, 53, 58). It thus will be critical to develop live-cell imaging sensors for these other pathways in order to gain a fuller understanding of how growth factors and other agents interactively regulate cell fate and functions under both physiological and pathological conditions. In the future, we envision that live-cell imaging studies with multiple readouts, coupled with other molecular and biochemical methods operating at the single cell level, should allow better understanding of how a diversity of signaling pathways are integrated in time and space to control specific outcomes in cells, tissues, and organisms.
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Disclosures

The authors declare that they have no conflicts of interest with the contents of this article.

Footnotes

1The abbreviations used are: EGF, epidermal growth factor; FBS, fetal bovine serum; HGF, hepatocyte growth factor; IGF-I, insulin-like growth factor-I; PDGF, platelet-derived growth factor; TGF-α, transforming growth factor alpha; SFM, serum-free medium.

Author contributions

S.M.G. and P.R. conceived of experiments, S.M.G. performed experiments; S.M.G. and P.R. interpreted results and wrote the manuscript.
References


**Figure Legends**

**Figure 1. Development of sensors for Erk2 and Akt activity.**  
**A. Left:** Top. Map of mKate2-Erk2 reporter molecule showing the positions of Erk2 phosphorylation sites at T185 and Y187; FP = fluorescent protein.  
**Bottom.** Diagram of the subcellular location of the mKate2-Erk2 reporter in cells with low growth factor signaling, where Erk2 is predominantly cytoplasmic, or high activity, where Erk2 is phosphorylated (P) and can be found in the nucleus.  

**Right:** Top. Map of FoxO1-clover sensor protein showing the positions of Akt phosphorylation sites at T24, S253, and S316.  
Also indicated are the nuclear localization sequence (NLS) and nuclear export sequence (NES) of FoxO1; FP = fluorescent protein.  
**Bottom.** Diagram of location of the FoxO1-clover reporter in cells with low growth factor signaling, where FoxO1 is predominantly nuclear, or high activity, where FoxO1 is highly phosphorylated (P) and is primarily cytoplasmic.

**B.** Time-lapse images of a representative experiment showing changes in the subcellular location of both reporter molecules in HeLa cells incubated with EGF [2.1 nM] for the times indicated.  
Scale bar = 50 μM.  

**C.** Time course of the relative nuclear intensity of the mKate2-Erk2 reporter in cells incubated in SFM and then exposed to SFM or EGF [2.1 nM] for 90 min.  
Population averages are presented in red (n = ~140 cells per incubation), with individual traces indicated in salmon (n = 10).  
The nuclear intensity of the reporter in each cell was normalized to its value at the start of imaging during incubation in SFM and scaled to the average peak EGF response.  

**D.** Time course of the relative translocation response of the FoxO1-clover reporter in the same cells as in C incubated in SFM and then exposed to SFM or EGF [2.1 nM] for 90 min.  
Population averages are presented (n = ~140 cells per incubation), with individual traces indicated in lime (n = 10).  
The nuclear intensity of the reporter in each cell was normalized to its value at the start of imaging during incubation in SFM and scaled to the average peak EGF response.  

**E.** Expression of phosphorylated Erk1/2 (pErk), total Erk, pRsk1/2, total Rsk, pAktS473 (pAkt), total Akt, pPRAS40, and total PRAS40, by immunoblotting using whole cell protein lysates from HeLa
cells after exposure to EGF for up to 90 min. Molecular mass markers are indicated to the right of each immunoblot.

**Figure 2.** Reporter dynamics after exposure of HeLa cells to different concentrations of EGF or HGF. **A, B.** Time course of the relative translocation responses of the mKate2-Erk2 reporter (top graphs) and the FoxO1-clover reporter (bottom graphs) in cells incubated in SFM and then exposed to different concentrations of EGF (left) or HGF (right) for 90 min. Population averages are presented ($n \approx 140$ cells per incubation per treatment group). For all experiments illustrated, the relative responsiveness of each reporter protein in each cell was normalized to values at the start of imaging during incubation in SFM and scaled to the average peak response.

**C.** Time course of the relative translocation response of the mKate2-Erk2 reporter (red traces) and the FoxO1-clover reporter (green) in individual cells incubated in SFM and then exposed to EGF [110 pM] (left panel) or HGF [572 pM] (right panel) for 90 min. For A-C, population averages are presented ($n \approx 140$ cells per incubation per treatment group).

**Figure 3.** Reporter dynamics after exposure of HeLa cells to different concentrations of growth factors. **A, B.** Time course of the relative translocation responses of the mKate2-Erk2 reporter (A) or the FoxO1-clover reporter (B) in cells incubated in SFM and then exposed to several concentrations of IGF-I for 90 min. Population averages are presented ($n \approx 140$ cells per incubation per treatment group). For all experiments illustrated, the relative responsiveness of each reporter protein in each cell was normalized to values at the start of imaging during incubation in SFM and scaled to the average peak response. **C.** Time course of the relative translocation response of the mKate2-Erk2 reporter (red traces) and the FoxO1-clover reporter (green) in individual cells incubated in SFM and then exposed to IGF-I [50 pM] for 90 min. **D, E.** Time course of the relative translocation response of the mKate2-Erk2 reporter (D) and the FoxO1-clover reporter (E) in cells incubated in SFM and then exposed to different growth factors for 90 min. Population averages are presented ($n \approx 140$ cells per incubation per treatment group).
treatment group). For all experiments pictured, the relative responsiveness of each reporter protein in each cell was normalized to values at the start of imaging during incubation in SFM and scaled to the average peak response. F. Dot plot of Erk and Akt activity, as indicated by responses of the mKate2-Erk2 reporter at 5 min and the FoxO1-clover reporter at 10 min in individual cells after incubation with SFM (black), TGFα ([1.67 nM], purple), EGF ([1.67 nM], blue), IGF-I ([250 pM], green), insulin ([1.0 nM], orange), or HGF ([1.72 nM], red) [n = ~150 cells per treatment group].

Figure 4. Reporter dynamics after exposure of HeLa cells to growth factors and different signaling inhibitors. A. Time course of the relative translocation response of the mKate2-Erk2 reporter (left graph) and the FoxO1-clover reporter (right graph) in cells incubated in SFM and then exposed to SFM or EGF [2.1 nM] ± different inhibitors for 90 min. B. Time course of the relative translocation response of the mKate2-Erk2 reporter (left graph) and the FoxO1-clover reporter (right graph) in cells incubated in SFM and then exposed to SFM or HGF [1.7 nM] ± different inhibitors for 90 min. C. Time course of the relative translocation response of the mKate2-Erk2 reporter (left graph) and the FoxO1-clover reporter (right graph) in cells incubated in SFM and then exposed to SFM or IGF-I [250 pM] ± different inhibitors for 90 min. For A-C, population averages are presented (n = ~140 cells per incubation per treatment group). For all experiments depicted, the relative responsiveness of each reporter protein in each cell was normalized to values at the start of imaging during incubation in SFM and scaled to the average peak response.

Figure 5. Reporter dynamics after exposure of HeLa cells to EGF and different signaling inhibitors. A. Time course of the relative translocation response of the mKate2-Erk2 reporter (left graph) and the FoxO1-clover reporter (right graph) in cells incubated in SFM and then exposed to SFM or EGF [2.1 nM] ± different inhibitors for 90 min. B. Time course of the relative translocation response of the mKate2-Erk2 reporter (left graph) and the FoxO1-clover reporter (right graph) in cells incubated in SFM and then exposed to SFM or IGF-I [250 pM] ± different inhibitors for 90 min.
reporter (right graph) in cells incubated in SFM and then exposed to SFM or EGF [2.1 nM] ± different inhibitors for 90 min. For A and B, population averages are presented (n = ~140 cells per incubation per treatment group). For all experiments shown, the relative responsiveness of each reporter protein in each cell was normalized to the values at the start of imaging during incubation in SFM and scaled to the average peak EGF response. C. Bar graph showing the average relative change in fluorescence intensity from an antibody array using HeLa cell lysates exposed to SFM or EGF [2.1 nM] ± different inhibitors for 15 min. Results are presented as mean ± SEM (n = 5 independent experiments). Phosphorylation sites are as follows: S6 ribosomal protein$^{\text{Ser235/236}}$, PRAS40$^{\text{Thr246}}$, p70 S6 kinase$^{\text{Thr421/Ser424}}$, Rsk1$^{\text{Ser380}}$, Erk$^{\text{Thr202/Tyr204}}$.

Figure 6. Erk activity negatively regulates Akt signaling by EGF. A. Time course of the relative translocation response of the FoxO1-clover reporter in C3H10T1/2 cells incubated in SFM and then exposed to SFM or EGF [2.1 nM] ± different inhibitors for 90 min. Population averages are presented (n = 50 cells per incubation). The relative responsiveness of the reporter protein in each cell was normalized to the value at the start of imaging during incubation in SFM and scaled to the average peak EGF response. B. Expression of pAkt, total Akt, pErk, and total Erk by immunoblotting using whole cell protein lysates from C3H10T1/2 cells after exposure to SFM or EGF plus the indicated inhibitors for 15 min. Molecular mass markers are indicated to the right of each immunoblot. C. Time course results for each of 25 individual cells incubated with EGF. D. Time course results for each of 25 individual cells incubated with EGF plus UO126 [10 μM]. E. Time course results for each of 25 individual cells incubated with EGF and PLX-4720 [10 μM]. Cells were imaged every 2 min during each treatment period. The nuclear intensity of the reporter in each cell was normalized to its value at the start of imaging during incubation in SFM and scaled to the average peak EGF response.

Figure 7. Erk activity negatively regulates Akt signaling by PDGF-AA. A. Time course of the relative translocation response of the FoxO1-clover reporter in C3H10T1/2 cells incubated in
SFM and then exposed to SFM or PDGF-AA [1.4 nM] ± different inhibitors for 90 min. Population averages are presented (\(n = 50\) cells per incubation). The relative responsiveness of the reporter protein in each cell was normalized to the value at the start of imaging during incubation in SFM and scaled to the average peak response. **B.** Time course results for each of 25 individual cells incubated with PDGF-AA. **C.** Time course results for each of 25 individual cells incubated with PDGF-AA plus UO126 [10 μM]. **D.** Time course results for each of 25 individual cells incubated with PDGF-AA and PLX-4720 [10 μM]. Cells were imaged every 2 min during each treatment period. The nuclear intensity of the reporter in each cell was normalized to its value at the start of imaging during incubation in SFM and scaled to the average peak response. **E.** Schematic of inter-relationships between the Ras – Raf – Mek – Erk, and PI3-kinase – Akt signaling pathways. Feedback inhibition by Erk activity on Raf and Ras, and activation of PI3-kinase by Ras are indicated. The locations of mKate2-Erk2 and FoxO1-clover as sensors for these pathways are shown.
Fig 1

A

growth factor activity:
Low
High

mKate2-Erk2

FoxO1-Clover

B

EGF: 0 5 10 15 30 60 90 min

mKate2-Erk2

FoxO1-Clover

C

mKate2-Erk2 Response (%)

EGF
SFM

FoxO1-Clover Response (%)

E

EGF [2.1 nM]

pErk
Erk
pRsk
Rsk
pAkt
Akt
pPRAS40
PRAS40

37 kDa

50 kDa

100 kDa
Fig 3

A

mKate2-Erk2 Response (%)

Time (min)

B

FoxO1-Clover Response (%)

Time (min)

C

IGF-I [50 pM]

Response (%)

Time (min)

D

mKate2-Erk2 Response (%)

Time (min)

E

FoxO1-Clover Response (%)

Time (min)

F

mKate2-Erk2 Response (%)

FoxO1-Clover Response (%)

Legend:

- mKate2
- clover
- HGF [1.72 nM]
- Insulin [1 nM]
- IGF-I [250 pM]
- EGF [1.67 nM]
- TGFα [1.67 nM]
- SFM

Concentration of IGF-I:

- 250 nM
- 125 nM
- 50 nM
- 25 nM
- 12.5 nM
- 0 nM

Concentration of SFM:

- 1.72 nM
- 1 nM
- 250 pM
- 1.67 nM
- 1.67 nM
- SFM

Time span:

0 to 90 minutes