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Graded Mitogen-Activated Protein Kinase Activity Precedes Switch-Like c-Fos Induction in Mammalian Cells

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The mitogen-activated protein kinase (MAPK) pathway is an evolutionarily conserved signaling module that controls important cell fate decisions in a variety of physiological contexts. During *Xenopus* oocyte maturation, the MAPK cascade converts an increasing progesterone stimulus into a switch-like, all-or-nothing response. While the importance of such switch-like behavior is widely discussed in the literature, it is not known whether the MAPK pathway in mammalian cells exhibits a switch-like or graded response. For this study, we used flow cytometry and immunofluorescence to generate single-cell measurements of MAPK signaling in Swiss 3T3 fibroblasts. In contrast to the case in *Xenopus* oocytes, we found that ERK activation in individual mammalian cells is not ultrasensitive and shows a graded response to changes in agonist concentration. Thus, the conserved MAPK signaling module exhibits different systems-level properties in different cellular contexts. Furthermore, the graded ERK response was converted into a more switch-like behavior at the level of immediate-early gene induction and cell cycle progression. Thus, while MAPK signaling is involved in all-or-nothing cell fate decisions for both *Xenopus* oocyte maturation and mammalian fibroblast proliferation, the underlying mechanisms responsible for the switch-like nature of the cellular responses are different in these two systems, with the mechanism appearing to lie downstream of the kinase cascade in mammalian fibroblasts.

Ultrasensitive switch-like responses control cell fate decisions in many biological settings, and the regulation of kinase activity is one way in which ultrasensitive behavior can be initiated (9, 14). Such behavior is characterized by an all-or-nothing response to increasing concentrations of stimulus (Fig. 1A). An ultrasensitive switch-like response displays a near vertical sigmoidal stimulus response curve. In the case of extreme ultrasensitive responses, signaling molecules switch between two discontinuous stable states and intermediate responses are not observed; this is referred to as a bistable response. Bistability may result from multistep phosphorylation events, positive feedback loops, double-negative feedback loops, and the relative abundance/activity of kinases and phosphatases driving the signaling cascade. In contrast to ultrasensitive signaling, a graded response in protein kinase activity changes incrementally with the stimulus concentration (Fig. 1B). Given the irreversible, all-or-nothing nature of many cell behaviors, including differentiation and cell cycle control, significant efforts have been focused on identifying the cellular mechanisms underlying bistability in biological systems.

Bistability in mitogen-activated kinase (MAPK) signaling was first demonstrated experimentally by the progesterone-induced maturation of *Xenopus laevis* oocytes. In pools of oocytes incubated with increasing concentrations of progesterone, extracellular signal-regulated kinase (ERK) activity appears to increase in a graded manner. However, when analyzed at the level of individual oocytes, the response of ERK is switch-like (9). When a total mammalian cell population is analyzed, ERK activation also appears to be graded. These studies with *Xenopus* oocytes suggest that ERK activation in individual mammalian cells may also exhibit bistability, but this behavior has not been observed due to population averaging and a lack of single-cell studies with a mammalian system. The goal of the present work was to analyze ERK activation in individual mammalian cells to address these systems-level properties of the MAPK pathway.

MATERIALS AND METHODS

Cell culture. Swiss 3T3 cells were maintained in Dulbecco's minimal essential medium (DMEM) containing 10% heat-inactivated fetal bovine serum. Swiss 3T3 cells (8×10^5) were cultured in 100-mm plates for 30 h and then deprived of serum for 40 h in DMEM-20 mM HEPES. Epidermal growth factor (EGF) and platelet-derived growth factor BB (PDGF-BB) were obtained from Invitrogen Corp. (Carlsbad, Calif.) and reconstituted in 0.1% bovine serum albumin (BSA). Swiss 3T3 cells stably expressing pMV-7-c-Fos were generated as described previously (13).

Cell lysis and immunoblotting. Cell extracts were prepared by washing cells in ice-cold phosphate-buffered saline (PBS) and harvesting them in lysis buffer (pH 7.2; 10 mM KPO_4, 1 mM EDTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 5 mM EGTA, 0.5% NP-40, 0.1% Brij 35, 1 mM sodium orthovanadate, 40 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 μg/ml pepstatin A). Extracts were centrifuged at 15,000 rpm for 10 min at 4°C, and cell lysates were immunoblotted by standard procedures with phospho-MEK1/2, phospho-GSK3, phospho-RSK1 (Cell Signaling Technologies), and anti-c-Fos (Upstate Biotechnology) antibodies. Antibodies directed against ERK1/2, p90 ribosomal S6 kinases (RSK), and phospho-325 c-Fos were previously described (13).

Flow cytometry and fluorescence-activated cell sorting (FACS) analysis. Swiss 3T3 cells (8×10^5) were cultured in 100-mm plates for 30 h, deprived of serum for 40 h in DMEM-20 mM HEPES, and treated with growth factors as indicated. The cells were trypsinized, fixed/permeabilized in ice-cold 100% methanol, washed in PBS, and stained intracellularly with phospho-ERK (Becton Dickinson Biosciences) and phospho-S473 Akt (Cell Signaling Technologies) for 1 h in 1% BSA. For anti-phospho-ERK1/2 analysis, a mouse monoclonal antibody recognizing the dually phosphorylated form of ERK1/2 (T202/Y204) was directly conjugated to Alexa Fluor 488. For phospho-Akt analysis, cells were washed with 1% BSA–PBS, incubated with Alexa Fluor 488-conjugated secondary antibodies for 1 h, washed, and resuspended in PBS. After being stained, the samples were analyzed by flow cytometry with a FACScan flow cytometer (Becton Dickinson), and data were acquired and analyzed with CellQuest software (Becton Dickinson

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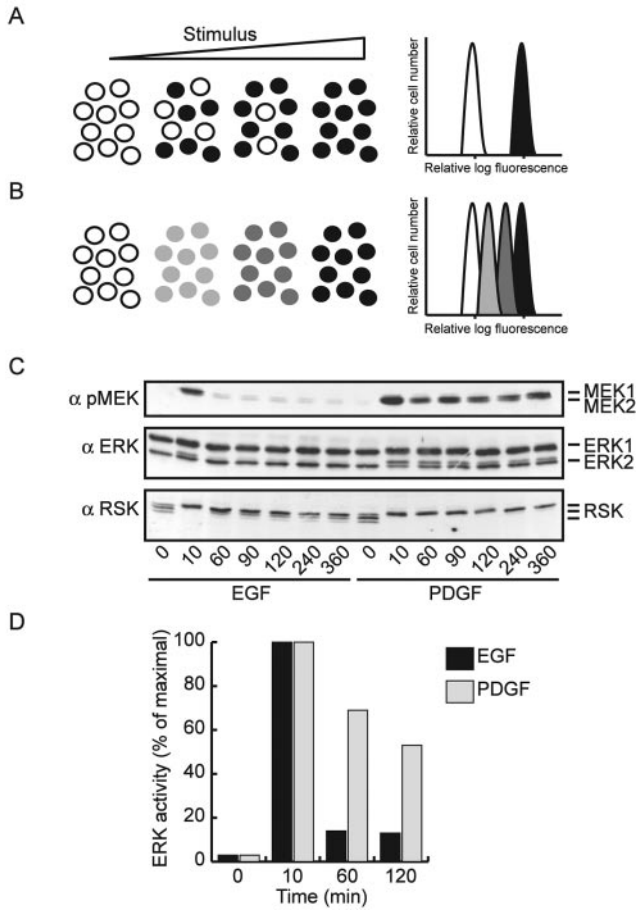

FIG. 1. ERK1/2 measurements of total cell populations. (A) Single cells exhibiting a bistable (all-or-nothing) response; (B) single cells exhibiting a graded response (linear). Quiescent Swiss 3T3 cells were treated with EGF (25 ng/ml) or PDGF (20 ng/ml) for the indicated times before total cell lysis. The activation kinetics of endogenous MEK1/2, ERK1/2, and RSK were analyzed by Western blotting (C) and an immune complex kinase assay (D).

Immunocytometry Systems). Geometric means were calculated to measure the central tendency of the fluorescent flow cytometric data.

In vitro kinase reactions. Cell extracts were immunoprecipitated with anti-ERK1 antibodies, and the ERK kinase activity was assayed in the presence of 10 μCi of [γ-^{32}P]ATP and 2 μg of recombinant GST-RSKD2/K464R as an in vitro substrate at 30°C for 10 min. Reactions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the amount of ^{32}P incorporated into GST-RSKD2/K464R was quantitated with a phosphorimager and ImageQuant software.

BrdU incorporation. Cells were induced with PDGF for 12 h, incubated with 20 μM bromodeoxyuridine (BrdU) for 15 min, and fixed in ice-cold 70% ethanol (1 h). To extract histones and denature the cellular DNA, we incubated the cells with 0.1 N HCl and 0.5% Triton X-100 (10 min), resuspended them in double-distilled water, incubated them at 100°C (10 min), and washed them with PBS containing 0.5% Triton X-100. For direct immunofluorescence staining, the cells were incubated for 30 min with an anti-BrdU fluorescein isothiocyanate-conjugated antibody (Becton Dickinson), washed in 0.5% Tween 20 plus 1% BSA in PBS, and resuspended in propidium iodide stain buffer (0.5% Tween 20, 250 μg/ml DNase-free RNase A, 20 μg/ml propidium iodide). After being stained, the samples were analyzed by flow cytometry with a FACScan flow cytometer (Becton Dickinson), and data were acquired and analyzed with CellQuest software (Becton Dickinson Immunocytometry Systems).

Immunocytochemistry. Swiss 3T3 cells were plated on poly-L-lysine-coated coverslips, serum starved, and treated with the indicated concentrations of PDGF for 10 min, followed by fixation in 3.7% paraformaldehyde in PBS for 10 min at room temperature. The cells were permeabilized with 0.2% Triton X-100 for 5 min. Immunostaining was performed by incubating cells with anti-dually phosphorylated ERK (1:500) monoclonal antibody (Sigma) or an anti-c-Fos (1:500) antibody (Upstate Biotechnology) followed by an Alexa 595- or fluorescein isothiocyanate-conjugated (1:500) antibody (Molecular Probes). DAPI (4′,6′-diamidino-2-phenylindole) was included in the mounting medium as a counterstain for nuclei and was visualized by photomicroscopy using an E800 Eclipse upright microscope (Nikon). For nuclear quantification of the c-Fos signal intensity, the images were individually analyzed with Metamorph software (Universal Imaging). Single nuclei were identified, and signals for integrated fluorescence intensity were collected. The cell number assessed for each concentration group ranged from 75 to 100. For scoring of c-Fos-positive nuclei, multiple fields for each concentration were imaged by using Metamorph software (Universal Imaging).

RESULTS AND DISCUSSION

Initially, we analyzed the ERK activation kinetics of a population of quiescent Swiss 3T3 fibroblasts treated with saturating concentrations of EGF or PDGF. Treatment with EGF or PDGF resulted in similar levels of endogenous phospho-MEK1/2 and mobility shifts of ERK/RSK indicating phosphorylation and activation at 10 min (Fig. 1C). However, a subsequent rapid inactivation accompanied the EGF treatment, lending a transient activation of MEK, ERK, and RSK that returned to basal levels at 60 min. In contrast, in PDGF-treated cells MEK, ERK, and RSK signaling was sustained beyond 360 min, and after 60 min the amount of phospho-MEK and ERK shifting was approximately half that observed at 10 min (Fig. 1C). Using immune complex kinase assays, we measured the maximal ERK1 phosphotransferase activities at 10 min post-EGF and -PDGF treatment (Fig. 1D). After 60 min of PDGF stimulation, ERK1 activation was ~60% of the maximal activation and remained ~50% activated beyond 120 min. At the same time points following EGF addition, ERK activity returned to a near-basal level.

Data from studies with *Xenopus* oocytes indicate that the MAPK cascades display an all-or-nothing response (2, 9) and have led to the generation of computational models stated to be widely applicable to cell signaling (1). However, these models have not been verified for kinase activation in mammalian cells at the single-cell level. To explore the biochemical responses of ERK in individual mammalian cells, we employed FACS-based technologies to assess the activation state of dually phosphorylated ERK within individual cells. The full activation of ERK occurs by phosphorylation on both threonine and tyrosine residues in the TEY motif of the activation loop, and ERK activation is correlated with dually phosphorylated ERK (3, 7, 16, 20). Using a phospho-specific antibody that recognizes dually phosphorylated ERK1 and ERK2, we found that the treatment of quiescent Swiss 3T3 fibroblasts with EGF (25 ng/ml) or PDGF (20 ng/ml) for 10 min produced similar levels of ERK activation, as measured by phospho-ERK flow cytometry (Fig. 2, blue and green histograms). When Swiss 3T3 cells were treated with PDGF for 60 min, we measured 60% ERK1 phosphotransferase activity in a whole cell population (Fig. 1D). If ERK activation exhibits true bistability, then the 60-min PDGF treatment would result in two distinct single-cell populations with cells in one of two stable states, either an off state or an on state, with no intermediate levels of ERK activation. Single-cell FACS measurements of phospho-ERK after 60 min of PDGF stimulation showed that individual cells in the

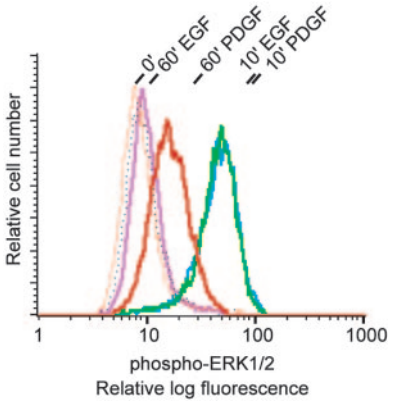

FIG. 2. Single-cell ERK1/2 measurements in mammalian fibroblasts. Serum-starved Swiss 3T3 cells were stimulated with either EGF (25 ng/ml) or PDGF (20 ng/ml) for 10 or 60 min. Single-cell ERK activities were measured by phospho-specific flow cytometry.

population showed intermediate levels of ERK activation (Fig. 2, red histogram) and therefore do not exhibit bistability.

To determine whether ERK activation in response to a growth factor treatment of mammalian cells is graded or all-or-nothing, we treated quiescent Swiss 3T3 cells with various (subsaturating) concentrations of PDGF for 10 min and used Western blot analysis to examine MEK and ERK activation (Fig. 3A). The stimulation of cells with increasing amounts of PDGF resulted in incremental MEK and ERK phosphorylation, indicative of a graded response. Immune complex kinase assays were then used to quantify these differences. Similar to ERK phosphorylation, ERK kinase activity also exhibited a graded response, increasing gradually from 66% to 100% (Fig. 3B). If ERK signaling in Swiss 3T3 cells were ultrasensitive, then subsaturating amounts of PDGF would drive single-cell responses which are all-or-nothing responses, with no intermediate levels of ERK activation. In contrast, if cells exhibited a graded response, then one would expect to detect intermediate levels of ERK activity. To discriminate between these two possibilities, we treated quiescent cells with 0.5, 1, 2, or 10 ng/ml PDGF for 10 min and measured the ERK1/2 activity by phospho-ERK flow cytometry (Fig. 3C). The maximum induction (10 ng/ml PDGF) shifted all cells to the on state (green histogram), while the 0.5- and 1-ng/ml doses (blue and yellow histograms, respectively) produced intermediate phospho-ERK states. The geometric mean fluorescent intensity values of phospho-ERK (Fig. 3C) correlated with the in vitro kinase data (Fig. 3B) in a PDGF dose-response curve. Extending these observations to another agonist, we activated Swiss 3T3 cells with EGF and used phospho-ERK flow cytometry to detect ERK1/2 kinase activation (Fig. 3D). Stimulation with 10 ng/ml EGF led to maximal ERK activation, while subsaturating concentrations of EGF (0.5, 1, 2, and 5 ng/ml) resulted in intermediate levels of ERK activation. Thus, at the single-cell level, Swiss 3T3 fibroblasts relay changes in agonist concentration to graded ERK activity.

We next examined whether additional pathways activated by PDGF exhibited a switch-like all-or-nothing response or a graded response. In the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, receptor activation leads to the recruitment of

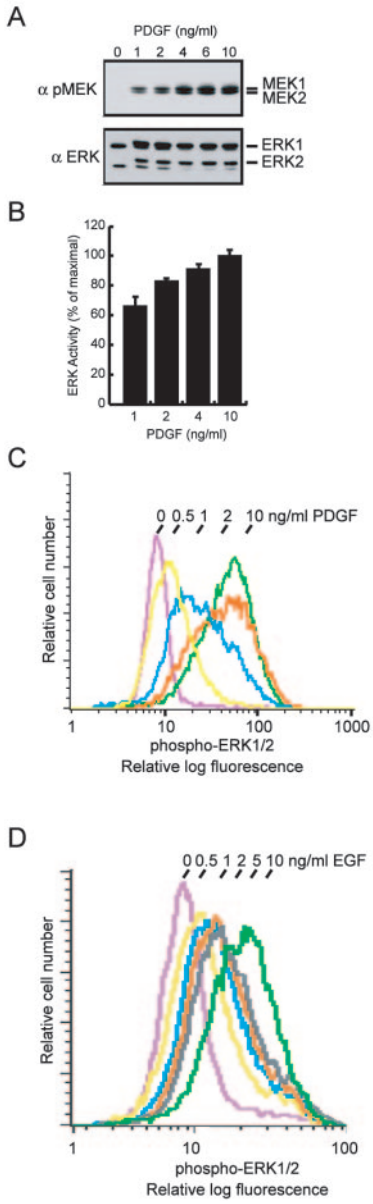

FIG. 3. Graded versus all-or-nothing ERK1/2 responses in mammalian fibroblasts. (A) Quiescent Swiss 3T3 cells were treated with PDGF (0, 1, 2, 4, 6, or 10 ng/ml) for 10 min before total cell lysis. The activation kinetics of endogenous phospho-MEK1/2 and total ERK1/2 were analyzed by Western blotting. (B) ERK1 was immunoprecipitated from a PDGF-treated population of cells, and the kinase activity was quantitated with an immune complex kinase assay. (C) Serum-starved Swiss 3T3 cells were stimulated with PDGF (0, 0.5, 1, 2, or 10 ng/ml). Single-cell ERK activities were measured by phospho-specific flow cytometry. (D) Serum-starved Swiss 3T3 cells were stimulated with a second agonist, EGF (0, 0.5, 1, 2, 5, or 10 ng/ml), and single-cell ERK activities were measured by flow cytometry.

PI3K lipid kinases to the plasma membrane, where PI3K phosphorylates lipids to form the second messengers phosphatidylinositol-3,4,5-triphosphate [$PI(3,4,5)P_3$] and $PI(3,4)P_2$ in response to extracellular agonists (5, 10, 11). The lipid products of PI3K activity facilitate the recruitment of Akt (protein kinase B) to the plasma membrane, where Akt is phosphorylated

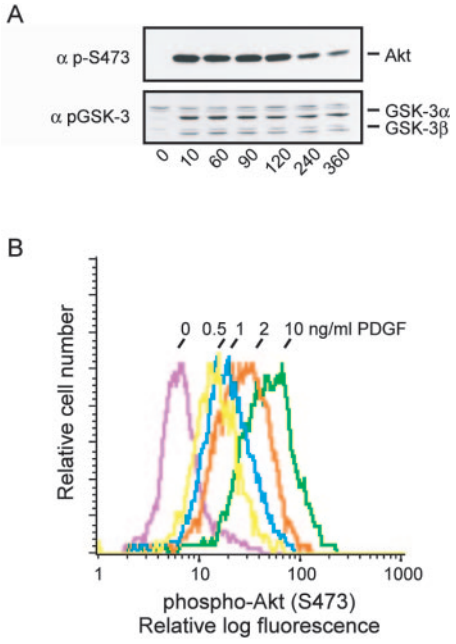

FIG. 4. Graded versus all-or-nothing Akt responses. (A) Quiescent Swiss 3T3 cells were treated with PDGF (20 ng/ml) for the indicated times before total cell lysis. The activation kinetics of endogenous Akt and GSK3 were analyzed by phospho-specific Western blotting. (B) Serum-starved Swiss 3T3 cells were stimulated with PDGF (0, 0.5, 1, 2, or 10 ng/ml) for 10 min, and single-cell AKT activities were measured by phospho-specific flow cytometry.

at Thr308 and Ser473, generating a fully phosphorylated and activated Akt kinase. To determine the kinetics of Akt activation and phosphorylation of the Akt target GSK3, we treated a population of cells with PDGF and analyzed Akt activation by Western blotting (Fig. 4A). Akt activation, as measured by Ser473 phosphorylation, was sustained maximally from 10 to 120 min, with a gradual rate of inactivation after 240 min. To test whether PDGF stimulation results in a graded or all-or-nothing response in the PI3K/Akt pathway, we treated Swiss 3T3 fibroblasts with PDGF (0.5, 1, 2, or 10 ng/ml) for 10 min and measured phospho-473 Akt in individual cells by flow cytometry (Fig. 4B). As the agonist concentration increased, the cells displayed a graded dose-dependent increase in Akt activation kinetics. Therefore, the activation of Akt is not bistable, or all-or-nothing, as intermediate dose-dependent Akt responses were observed with 0.5, 1, and 2 ng/ml PDGF. Interestingly, the activation of Akt in response to subsaturating PDGF concentrations was more gradual that that of ERK (compare Fig. 4B to Fig. 3C).

ERK signaling can regulate fundamental aspects of cellular behavior, such as cell cycle progression and proliferation, that exhibit switch-like all-or-nothing behavior. In order to quantitatively analyze the entry into S phase that accompanies graded ERK signals in single cells, we used flow cytometry to measure BrdU incorporation following PDGF treatment (Fig. 5A). BrdU quantitation showed that the graded ERK activity at 1 ng/ml (Fig. 3B) did not induce cells to enter S phase, while 10 ng/ml increased BrdU incorporation 10-fold, indicating that cell cycle progression requires a minimum threshold of ERK activation (Fig. 5A). Since ERK signaling regulates S-phase entry in Swiss 3T3 cells (13), how does a graded ERK signal result in a cell proliferation response? One possibility is that subtle differences in ERK activity somehow result in quantitative differences in the induction of genes, such as immediate-early genes, which are required for cell cycle progression. To explore this possibility, we incubated quiescent Swiss 3T3 fibroblasts with 1, 5, or 10 ng/ml PDGF and assayed the induction of c-Fos, the protein product of the c-*fos* immediate-early gene (Fig. 5B). The PDGF-regulated expression of c-Fos in Swiss 3T3 cells was dependent on ERK signaling (data not shown). Although the levels of c-Fos induced after 60 min were similar in cells treated with 5, 10, or 20 ng/ml PDGF, little, if any, c-Fos was detected in cells treated with 1 ng/ml, even though this dose led to 66% ERK activity. To examine individual cells for switch-like c-Fos induction, we treated Swiss 3T3 fibroblasts with increasing concentrations of PDGF (0.5, 1, 2, and 5 ng/ml) and measured the c-Fos levels at 60 min by immunofluorescence with antibodies specific for c-Fos (Fig. 5C). c-Fos induction was ultrasensitive, with no response at concentrations of 0.5 and 1 ng/ml and with all cells responding at 2 ng/ml PDGF. This ultrasensitive switch-like behavior could be quantified by using integrated nuclear c-Fos fluorescent intensities (Fig. 5C). The measured differences in c-Fos induction were significant and support the finding that graded ERK activity precedes c-Fos ultrasensitivity. In addition, the PDGF stimulus response curve had a sharp, almost step-like, function from 1 to 2 ng/ml PDGF (Fig. 5D). Previously, we have shown that subtle differences in ERK signal duration after the initial treatment translate into substantial differences in Fos phosphorylation, stability, and BrdU incorporation (12). In accordance with our BrdU analysis, a minimum threshold of ERK activation was required for c-*fos* induction. Taken together, these results demonstrate that c-*fos* induction is highly ultrasensitive, despite the absence of any ultrasensitive ERK signals upstream.

One possible way that graded ERK activation results in switch-like downstream effects on gene expression and S-phase entry is through the regulation of ERK nuclear translocation. Active ERK1/2 has both cytoplasmic and nuclear targets. Among the ERK-specific substrates, RSK1 is predominantly a cytoplasmic target, while c-Fos is exclusively a nuclear target. Previously, we have shown that ERK phosphorylates Thr325 in c-Fos and that this phosphorylation is tightly associated with nuclear ERK activity (13), whereas cytoplasmic ERK phosphorylates RSK1 Thr573, which is located in the C-terminal kinase activation loop (15). To examine a cytoplasmic target, we treated cells with subsaturating concentrations of PDGF and measured endogenous RSK1 Thr573 phosphorylation. The stimulation of Swiss 3T3 cells with 0.5, 1, and 2 ng/ml PDGF resulted in robust RSK1 phosphorylation but undetectable phosphorylation of stably expressed c-Fos (Fig. 6A). In fact, the phosphorylation of c-Fos required concentrations of >2 ng/ml PDGF and was more ultrasensitive than that of RSK. This observation, along with the switch-like c-Fos induction, supports the possibility that bistability in mammalian cells occurs at the level of ERK1/2 nuclear translocation.

The translocation of activated ERK into the nucleus is essential for transcription factor phosphorylation, target gene induction, and cell cycle progression (4, 6). It has been suggested that translocation events can generate ultrasensitive

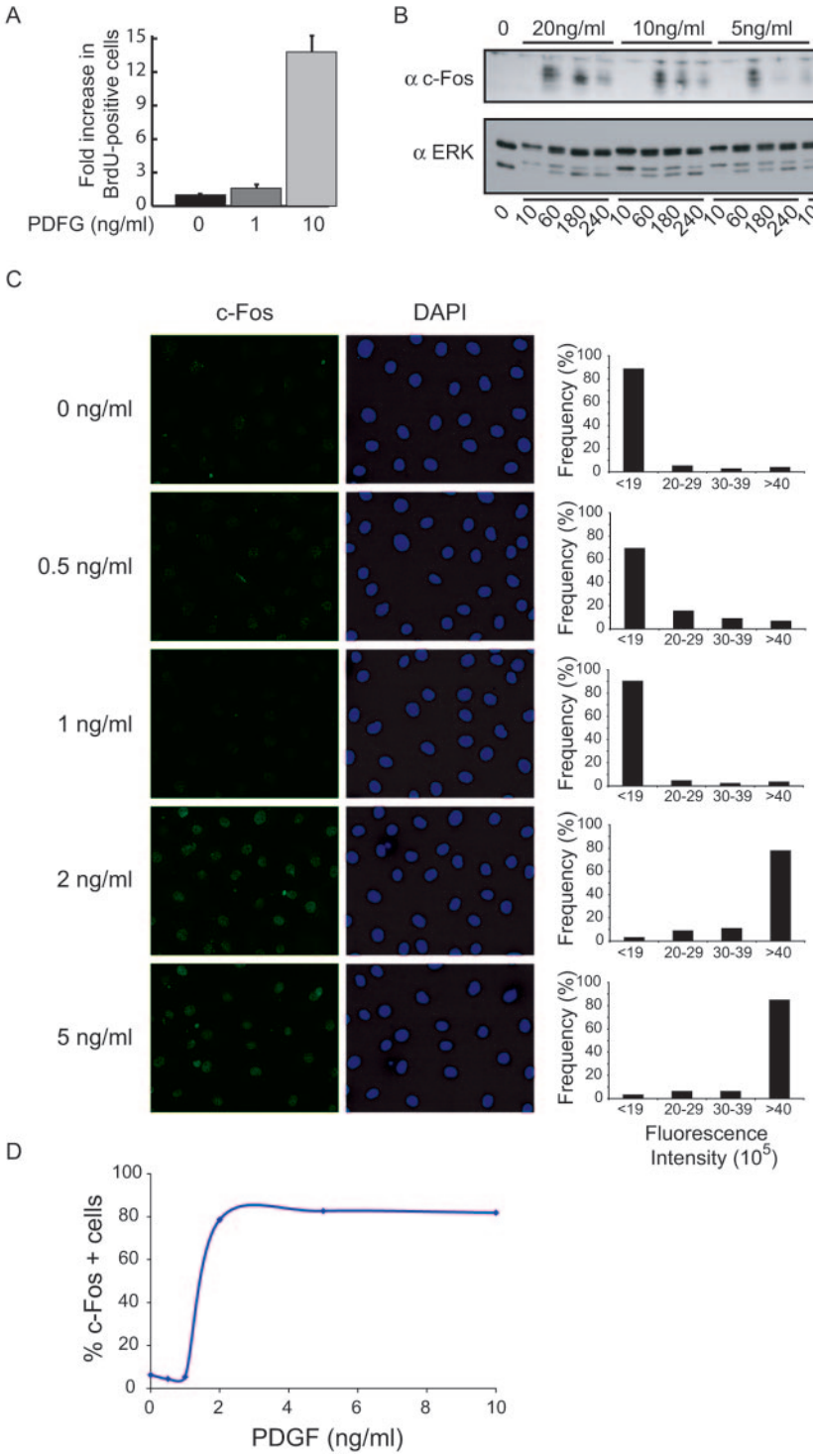

FIG. 5. Graded ERK activity leads to switch-like c-Fos induction and cell proliferation. (A) Quiescent Swiss 3T3 cells were induced with PDGF for 12 h, treated with BrdU (20 μM) for 15 min, and then fixed. The number of BrdU-positive cells was quantitated by FACS analysis. (B) Quiescent Swiss 3T3 cells were treated with PDGF (20, 10, 5, or 1 ng/ml) for the indicated times before total cell lysis. The levels of endogenous c-Fos and the activation kinetics of ERK1/2 were measured by Western blotting. (C) Serum-starved Swiss 3T3 cells were stimulated with PDGF (0, 0.5, 1, 2, or 5 ng/ml) for 60 min. c-Fos induction was monitored by epifluorescence (left panels), and DAPI was used to stain nuclei (right panels). Single-cell fluorescent intensity quantification for the nucleus (graphs) revealed switch-like c-Fos induction from 1 ng/ml to 2 ng/ml PDGF. Representative images of c-Fos are shown, together with nuclear fluorescence intensities, for the indicated concentrations of PDGF. (D) Stimulus response curve illustrating an ultrasensitive switch-like response from 1 to 2 ng/ml PDGF. After stimulation with PDGF for 60 min, the percentages of c-Fos positive cells were determined with Metamorph software. The data were derived from multiple experiments with 75 to 100 cells per condition.

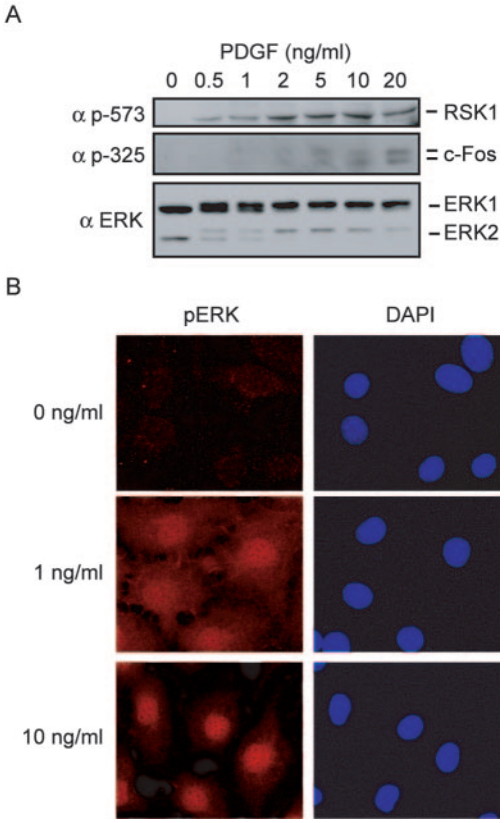

FIG. 6. Subcellular localization of activated ERK. (A) Quiescent Swiss 3T3 cells were treated with PDGF (0, 0.5, 1, 2, 5, 10, or 20 ng/ml) for 10 min before total cell lysis. The kinetics of endogenous RSK and stably expressed c-Fos phosphorylation and total ERK1/2 levels were analyzed by Western blotting. (B) Serum-starved Swiss 3T3 cells were stimulated with PDGF (0, 1, or 10 ng/ml) for 10 min. ERK activation (pERK) and localization were monitored by epifluorescence (left panels), and DAPI was used to stain nuclei (right panels).

signaling in vivo (8). To examine the distribution of active ERK in PDGF-treated cells, we used indirect immunofluorescence. The treatment of cells with 10 ng/ml PDGF (Fig. 6B) resulted in almost exclusively nuclear localization of active ERK, as noted previously (13). Surprisingly, the distribution of active ERK in cells treated with a low dose of PDGF (1 ng/ml) was both nuclear and cytoplasmic (Fig. 6B). As expected, we did not detect phosphorylated ERK in quiescent Swiss 3T3 cells. These observations suggest that the agonist concentration affects the subcellular localization of activated ERK, which in turn controls signal specificity. We suggest that incremental changes in graded ERK activity alone cannot account for ultrasensitive responses in mammalian cells. Instead, currently unknown mechanisms that control nuclear translocation and/or retention are likely responsible for altering the localization of ERK activity, resulting in ultrasensitive responses downstream.

In summary, these findings reveal that at the level of a single cell, ERK exhibits a graded response to growth factor stimulation. Our results are in agreement with a recent report by Whitehurst et al., who showed graded ERK1/2 responses in primary human cells (18), and taken together provide solid evidence of graded ERK signaling in mammalian cells. These results contrast with progesterone-induced *Xenopus* oocyte maturation, for which ERK activation is bistable. Thus, in different cellular contexts, the MAPK cascade clearly exhibits different systems-level properties. Sensing of the signal strength at the membrane initiates changes in the physiological state of the cell, from small-scale alterations in gene expression or changes in phosphorylation states of kinases to large-scale changes in cellular differentiation, mitosis, or apoptosis. The occurrence of intermediate ERK activation raises mechanistic questions regarding the importance of thresholds at the cellular, organ, and whole-organism levels. Another example of a protein that may sense MAPK signal strength is p21Cip1. Low to intermediate levels of RAF activation promote cell cycle progression, whereas high levels promote p21Cip1-dependent cell cycle arrest (17, 19). Importantly, we found that the proliferative response to growth factor stimulation of Swiss 3T3 cells is switch-like, and we propose that the ultrasensitive step in the MAPK pathway is downstream of ERK activation, at the level of ERK nuclear concentration and c-Fos induction, demonstrating that different cellular responses that engage the MAPK pathway exploit different mechanisms to achieve an ultrasensitive response. Although ERK-regulated cell cycle entry in mammalian cells is switch-like, intermediate ERK activation could have an important role in homeostasis and cell survival since many ERK targets regulating these processes are localized in the cytoplasm. These results and other single-cell measurements of kinase activity and nuclear accumulation should allow for computational models of signal transduction and gene induction to be developed for mammalian cells. Ultimately, such models may help to guide drug discovery by allowing research to focus on therapeutic strategies for particular aspects of a signaling network that are most relevant to a particular cellular event.

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