Spatial analysis of key signaling proteins by high-content solid-phase cytometry in Hep3B cells treated with an inhibitor of Cdc25 dual-specificity phosphatases.

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Summary

Protein phosphorylation frequently results in the subcellular redistribution of key signaling molecules, and this spatial change is critical for their activity. Here we have probed the effects of a Cdc25 inhibitor, 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone, or Compound 5, on the spatial regulation and activation kinetics of tyrosine phosphorylation-dependent signaling events using two methods: i) high-content, fluorescence-based, automated solid phase cytometry and ii) a novel cellular assay for Cdc25A activity in intact cells. Immunofluorescence studies demonstrated that Compound 5 produced a concentration-dependent nuclear accumulation of phospho-Erk and phospho-p38, but not NFkB. Immunoblot analysis confirmed Erk phosphorylation and nuclear accumulation, and in vitro kinase assays showed that Compound 5-activated Erk was competent to phosphorylate its physiological substrate, the transcription factor Elk-1. Pretreatment of cells with the MEK inhibitor U-0126 prevented the induction by Compound 5 of nuclear phospho-Erk, but not phospho-p38 accumulation, and protected cells from the antiproliferative effects of Compound 5. Overexpression of Cdc25A in whole cells caused dephosphorylation of Erk that was reversed by Compound 5. The data show that an inhibitor of Cdc25 increases Erk phosphorylation and nuclear accumulation and support the hypothesis that Cdc25A regulates Erk phosphorylation status.
Introduction

Approximately one third of mammalian proteins are thought to be posttranslationally modified by phosphorylation (1). The human genome contains hundreds of protein kinases (2), but the reversibility of the phosphorylation process suggests that phosphatases also play a major role in the regulation of protein phosphorylation. While the roles and cellular functions of kinases have been extensively studied, protein phosphatases have received much less attention. A long held view has been that phosphatases merely serve to reverse the actions of protein kinases. More recently it has been recognized that phosphatases may be as numerous and as tightly regulated as protein kinases, with as widely varying substrate specificities and signaling functions (3). The preference of certain phosphatases for one phosphorylated hydroxy amino acid over others has resulted in the current classification of phosphatases as serine/threonine specific (STPase)$^1$, tyrosine-specific (PTPases), and dual-specific (DSPases) phosphatases. While several highly potent and selective inhibitors of STPases have been isolated from natural sources, selective PTPase or DSPase inhibitors are still rare.

Protein kinases and phosphatases are part of a complex signaling network of tightly regulated dynamic processes. The nature and details of network organization are just beginning to be unveiled, but their abundance, diversity, and substrate specificity alone cannot explain how these molecules function to regulate complex biochemical pathways. An emerging concept in signaling specificity is the subcellular location at which signaling events occur (4). Most protein movements within the cell are consistent with a random diffusion process. However, it is now being recognized that spreading as well as
restriction of signaling events to certain regions of the cell is driven by the availability of sites for protein-protein and protein-second messenger interactions (5). Phosphorylation of many key signaling molecules causes a subcellular redistribution that is critical for biological activity (6-8).

Despite the potential importance that spatial regulation might have in signal transduction and the considerable information that can be derived from localization studies, a lack of readily available, quantitative analytical tools to assess the subcellular localization of multiple signal transduction molecules has impeded progress in this area. Current fluorescent imaging techniques have low-throughput and are not well suited for the dissection of how complex signaling networks are coordinated. In this report, we have used a novel, automated, fluorescence-based, multiparametric, solid-phase cytometer, the Cellomics ArrayScan II (9) to rapidly quantitate the effects of a synthetic K vitamin analog, Compound 5, on the spatial regulation of a subset of key signal transduction molecules.

Compound 5 was discovered to be a potent inhibitor of hepatoma cell growth in a small targeted library of synthetic K Vitamin analogs (10, 11). It has antiphosphatase activity that is thought to contribute to its antiproliferative activity. Most notably, it is one of the most potent in vitro inhibitors of the Cdc25 phosphatase family of dual-specificity phosphatases reported to date (12). In vitro, Compound 5 is approximately 10- and 100-fold more potent against Cdc25 compared with the prototype DSPase VHR, or protein tyrosine phosphatase 1B (PTP1B), respectively (12). Its ability to cause a dual cell cycle
arrest in G1 and G2 phase as well as increased phosphorylation of the Cdc25 substrates, Cdc2 (Cdk1), Cdk2, and Cdk4, are consistent with Cdc25 phosphatase inhibition by Compound 5 (12). Recent work from our laboratories has also demonstrated that Compound 5 causes increased tyrosine phosphorylation on a number of proteins, including the epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase (Erk) in hepatocytes (13) and MCF-7 cells (14), but it is unknown how Compound 5 enhanced Erk phosphorylation or whether Compound 5 treatment changed Erk subcellular localization. A possible link between the mitogenic signal transduction and Cdc25A has been described by Galaktionov et al (15), who reported that Cdc25A associates with Raf-1, a key upstream activator of Erk, in mammalian cells and frog oocytes. More recently, evidence for a possible functional involvement of Cdc25A in the Erk pathway was presented by Xia et al. (16), who reported that co-expression of Cdc25A, together with Raf-1, prevented Raf-1 activation in response to PDGF in NIH3T3 cells. Nonetheless, no direct evidence for Cdc25A involvement in Erk phosphorylation or activity has been reported.

In the current study, we demonstrated that Cdc25A expression could reduce Erk phosphorylation, and described a novel cell-based assay revealing that Compound 5 directly interfered with Cdc25A function on Erk phosphorylation. Using quantitative, fluorescence-based, solid phase cytometry, we documented that Erk hyperphosphorylation by Compound 5 resulted in increased nuclear accumulation of kinase-active phospho-Erk. Thus, an inhibitor of Cdc25 increased Erk phosphorylation,
which further supported the hypothesis that Cdc25A regulates Erk phosphorylation status.
Experimental Procedures

**Reagents.** Compound 5, 2-(2-mercaptopethanol)-3-methyl-1,4-naphthoquinone, has been described previously (10). Human recombinant interleukin 1-α (IL-1α) was from R&D systems (Minneapolis, MN). Mouse monoclonal anti-phospho-Erk antibody (E10) and the MEK inhibitor U-0126 were from New England Biolabs (Beverly, MA). Mouse monoclonal Erk 2 antibody was from Upstate Biotechnology (UBI, Lake Placid, NY). Primary antibodies for phospho-p38 and the p65 subunit of NFkB were components of a commercially available assay kit (Cellomics, Pittsburgh, PA). Anti-Oct-1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-HSP90 antibody was from BD Transduction Laboratories (San Diego, CA). Secondary antibodies were Alexa-Fluor 488 conjugated goat anti-mouse (p-Erk), goat anti-rabbit (p38, p-JNK), or donkey anti-goat (NFkB) IgG (Molecular Probes, Eugene, OR).

**Cell culture.** Cells were maintained in Dulbecco’s Minimum Essential Medium (DMEM) containing 10% fetal bovine serum (FBS, HyClone, Logan, UT), and 1% penicillin-streptomycin (Life Technologies, Inc., Rockville, MD) in a humidified atmosphere of 5% CO₂ at 37°C. HeLa, PC-3, DU-145, and NIH 3T3 cells were from ATCC. Rat-1 fibroblasts were obtained from Dr. Guillermo Romero, University of Pittsburgh. Hep3B human hepatoma cells have been characterized previously (17).

**Indirect immunofluorescence.** Hep3B, HeLa, PC-3, DU-145, Rat-1, or NIH 3T3 cells (4,000 cells per well) were plated in the wells of a collagen-coated 96 well darkwell plate
(Packard ViewPlate™), and allowed to attach overnight. Cells were treated for the times indicated with Compound 5 or IL-1α, fixed with 3.7% formaldehyde in PBS and permeabilized with PBS/Triton X-100. Cells were stained with antibodies against phospho-Erk, phospho-p38, phospho-JNK, or the 65 kDa subunit of NFkB, and washed with PBS/Tween20. Nuclei were stained with Hoechst 33342 fluorescent dye, and immunoreactive cells were visualized by AlexaFluor 488 secondary antibodies (Molecular Probes, Eugene, OR) using an XF100 filter set at excitation/emission wavelengths of 494/519 nm (Alexa 488), and 350/461 nm (Hoechst), respectively. Plates were analyzed by automated image analysis on the ArrayScan II system (Cellomics, Pittsburgh, PA) using the previously described cytoplasm-to-nuclear translocation algorithm (18). Control experiments omitting primary antibodies were performed each time to assess the amount of non-specific background staining.

**Cell Fractionation and Western Blotting.** Cytosolic and nuclear fractions were prepared using a slightly modified procedure as published by Schreiber et al. (19). Hep3B cells were plated in 100 mm tissue culture dishes, exposed to 10 µM Compound 5 for the indicated periods of time, and harvested by centrifugation. Cell pellets were resuspended in 200 µl of hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, and 0.5% Nonidet-P-40), incubated on ice for 10 min, disrupted by repeated aspiration through a 20-gauge needle, and centrifuged at 2,500 x g for 15 min. The supernatant was collected as cytosolic extract. Nuclear pellets were resuspended in nuclear extraction buffer (20 mM HEPES, pH 7.9, 10% glycerol, 1.5 mM MgCl₂, 400 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT), incubated on
ice for 1 h, and centrifuged at 13,000 x g to collect the nuclear fraction. Solubilized proteins were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (NEN, Boston, MA). Membranes were probed with anti-phospho-Erk, anti-Oct-1, and anti HSP-90 antibodies. Positive antibody reactions were visualized using peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) and an enhanced chemiluminescence detection system (Renaissance, NEN, Boston, MA) according to manufacturer’s instructions.

**Erk activity assay.** Erk activity in cytosolic and nuclear fractions was determined using a non-radioactive immunoprecipitation kit (Cell Signaling Technologies, Beverly, MA). Briefly, 200 µg of nuclear or cytosolic proteins were incubated with 15 µl of agarose-conjugated anti-phospho-Erk antibody, and incubated overnight at 4°C with gentle rocking. Immunoprecipitates were pelleted and washed twice with kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na₃VO₄, and 10 mM MgCl₂). Pellets were resuspended in 50 µl of kinase buffer supplemented with 200 µM ATP and 2 µg of Elk-1 GST fusion protein, and incubated for 30 min at 30°C. Immunoprecipitates were boiled in SDS-PAGE sample buffer and analyzed by Western blot using an anti-phospho-Elk-1 antibody.

**Growth inhibition assay.** The antiproliferative activity of Compound 5 in combination with the MEK inhibitor, U-0126, was measured by a previously described assay based on fluorimetric quantitation of total cellular DNA content using the fluorochrome Hoechst 33258 (20). Briefly, cells were grown in 96 well microplates and treated every day with
for three days with various concentrations of Compound 5 in the presence or absence of the MEK inhibitor, U-0126 (5 µM). Cells were lysed by repeated freeze-thawing and cellular DNA was quantitated as described (20).

**Cell transfections.** Mammalian expression plasmids encoding full length wild-type and catalytically inactive C430S mutant Cdc25A in a pcDNA3 vector were generously provided by Dr. Thomas Roberts, Dana Farber Cancer Institute (16). Transfections were carried out by the Lipofectamine method as per manufacturer’s instructions (Life Technologies, Rockville, MD). Briefly, HeLa cells (100,000 per well) were plated in the wells of a six well plate and transfected with 0.5 µg of cDNA in OPTI-MEM transfection medium using Lipofectamine Plus™ reagent (Life Technologies, Rockville, MD). Three hours after transfection, the medium was replaced with complete growth medium and the cells were allowed to recover for 48 h. Cells were treated with 0-20 µM Compound 5 for 30 min and protein lysates were prepared and analyzed by SDS-PAGE and Western Blot analysis for phospho-Erk and Erk 2 levels as described above. For quantitation of protein expression levels, X-ray films were scanned on a Molecular Dynamics personal SI densitometer and analyzed using the ImageQuant software package (Ver. 4.1, Molecular Dynamics, Sunnyvale, CA).
Results

A fluorescence-based high-content assay for phospho-Erk nuclear accumulation.

Compound 5 was previously found to induce the prolonged phosphorylation of tyrosines on a number of signaling proteins in the Erk cascade, including Erk1 and Erk2 (13, 14). We first asked whether this increase in tyrosine phosphorylation was associated with a change in phospho-Erk nuclear accumulation. Hep3B cells were either incubated with vehicle (DMSO) (Fig. 1A-C) or Compound 5 (Figure 1D-F) for 30 min and immunostained with antibodies against a dually phosphorylated (Thr202/Tyr204) form of Erk (Fig. 1B,C,E,F). Nuclei were visualized by Hoechst 33342 staining (Fig. 1A and D). Figure 1B shows that vehicle-treated cells had very low levels of phospho-Erk, most of which was diffusely distributed in the cytoplasm. Treatment of cells with Compound 5 resulted in a substantial increase in total phospho-Erk, with prominent nuclear accumulation (Fig. 1E). Overlay images (Fig. 1C and F) illustrate the quantitation of cytoplasmic and nuclear phospho-Erk levels. Fluorescence-labeled cells were analyzed in two separate channels by the ArrayScan II and the cytoplasm-to-nuclear distribution determined by a previously described algorithm (18). Hoechst 33342 staining (Fig. 1A and D) defined the nuclear area. Phospho-Erk fluorescence intensity within this nuclear area was referred to as “cytonuclear intensity”. To assess the amount of fluorescently labeled phospho-Erk in the cytoplasm, a set of concentric rings spaced by two pixels was placed around the nuclear boundary. Phospho-Erk fluorescence intensity within the ring area was referred to as “cytoring intensity”. Both cytonuclear and cytoring intensities were normalized to the total cytonuclear or cytoring area, and are expressed as average
intensity per pixel. All cytoplasmic-to-nuclear difference values were calculated by subtracting the average cytoplasmic intensity per pixel from the average cytonuclear intensity per pixel. Thus, an increase in the cytonuclear difference value is indicative of Erk activation through phosphorylation, translocation, or both.

**Induction of phospho-Erk and phospho-p38, but not NFκB, by Compound 5.** We next examined whether Compound 5 caused selective nuclear Erk accumulation by comparing its effects to those of other signaling events, which have also been reported to be activated in a tyrosine phosphorylation-dependent manner, and are thought to mediate stress responses. Cells were treated for 30 min with either 10 µM Compound 5 or 25 ng/ml interleukin-1 alpha (IL-1α), immunostained with phospho-Erk, phospho-p38, phospho-JNK, or p65 NFκB antibodies, respectively and analyzed for differences in cytoplasmic-to-nuclear fluorescence intensity. A total of 100 cells were imaged in each well. Figure 2 shows that Compound 5 lead to a dramatic increase in nuclear accumulation of phospho-Erk and phospho-p38, but had only a moderate effect on phospho-JNK, and did not affect the nuclear accumulation of NFκB. IL-1α, in contrast, activated all three stress-response mediators (p38, JNK, and NFκB), but not Erk. Thus, the activity profile of Compound 5 was distinct from that of the cytokine IL-1α, suggesting that Compound 5 was not a general stress-inducing agent.

**Kinetics of Erk and p38 activation by Compound 5.** Experiments with the stress inducer and phosphatase inhibitor, sodium arsenite, previously demonstrated that p38 and Erk were activated with different kinetics in a variety of cell lines (21). These authors
also reported that Erk activation was abrogated by dominant negative forms of p38 and the p38 specific kinase inhibitor, SB-203580, suggesting an involvement of p38 in Erk activation. We thus examined concentration-dependence and kinetics of phospho-Erk and phospho-p38 activation in Hep3B cells. Figure 3 shows that maximal stimulation of both Erk and p38 was obtained at 10 µM Compound 5. Moreover, continuous exposure to 10 µM Compound 5 caused a progressively greater activation and nuclear accumulation with similar temporal characteristics (Figure 3B). We have also found that the p38 inhibitor SB-203580 did not inhibit phospho-Erk nuclear accumulation (data not shown). These results suggest that Compound 5 acted differently than the nonspecific tyrosine phosphatase inhibitor sodium arsenite.

**Irreversibility of Compound 5 action.** Compound 5 is a sulfhydryl-arylating agent and its sustained antiphosphatase activity has been ascribed to covalent modification of critical cysteine residues on dual-specific and tyrosine phosphatases (17). To test whether its effects were irreversible, we treated cells with Compound 5 for 5 or 10 min, followed by washout, and compared the magnitude of phospho-Erk and phospho-p38 accumulation to that obtained after a 30 min continuous exposure. Figure 4 shows that short pulses of Compound 5 resulted in substantial activation of both Erk and p38, consistent with a rapid and persistent inhibition of cellular phosphatases after compound removal.

**Biochemical analysis confirms phospho-Erk nuclear accumulation.** We next validated the results from the automated fluorescence-based analysis by conventional
biochemical methods. Cells were treated with 10 µM Compound 5 for the indicated times, lysed, separated into cytosolic and nuclear fractions, and analyzed by Western blot using a phospho-Erk antibody (Figure 5A). Untreated cells had almost no nuclear phospho-Erk, consistent with the whole cell images in Figure 1B. Within minutes, Compound 5 caused a time-dependent and sustained increase in nuclear phospho-Erk accumulation. In contrast, cytosolic phospho-Erk levels in control cells were higher than those in the nucleus and increased only after a longer exposure to Compound 5 (30 min, Figure 5A). The results from the immunoblot analysis thus confirmed those from the less arduous solid phase cytometry studies.

**Phosphorylated Erk from Compound 5 treated cells is activated and phosphorylates Elk-1.** We then used the identical lysates from Compound 5 treated cells to investigate whether the observed Erk phosphorylation resulted in an increase in Erk kinase activity. It is thought that upon phosphorylation by MEK1 and MEK2 in the cytosol, a fraction of Erk translocates to the nucleus, where it phosphorylates and activates transcription factors, such as c-fos, c-jun, and Elk-1 (22). To investigate whether phosphorylated Erk was functional in Compound 5-treated cells, we examined its ability to phosphorylate the transcription factor, Elk-1. Phospho-Erk was immunoprecipitated from Compound 5 treated and untreated cells and immunoprecipitates were subjected to an *in vitro* kinase assay using recombinant GST-Elk-1 fusion protein as a substrate. Assay mixtures were separated on SDS-PAGE and immunoblotted with an anti-phospho-Elk-1 antibody. Figure 5B shows that nuclear phospho-Erk had kinase activity and that its kinetics of activation correlated well with its phosphorylation status. Compound 5-induced nuclear
phospho-Erk was thus functional and able to phosphorylate its physiological substrate, Elk-1.

**MEK inhibition and nuclear translocation of phospho-Erk, phospho-p38 or phospho-JNK.** We next investigated possible consequences of Erk or p38 activation by Compound 5. We first examined whether inhibition of MEK, the direct upstream activating kinase for Erk, would reduce phospho-Erk nuclear accumulation. Cells were pretreated with the MEK1/MEK2 inhibitor U-0126 (23) for 45 min, stimulated with Compound 5 for an additional 30 min in the presence of the inhibitor, and analyzed on the ArrayScan II for nuclear accumulation of phospho-Erk, phospho-p38, and phospho-JNK. Consistent with results from Figure 2, Compound 5 caused a robust increase in nuclear phospho-Erk and phospho-p38, but had only a partial effect on phospho-JNK (Figure 6). Inclusion of 10 µM U-0126 caused almost complete inhibition of Compound 5-induced Erk activation, but, as expected, had little or no effect on p38 or JNK activation. These data suggest that MEK inhibition is sufficient to inhibit phospho-Erk nuclear accumulation by Compound 5.

**The MEK inhibitor U-0126 protects cells from the antiproliferative effects of Compound 5.** To determine whether the activation of Erk or p38 played a role in mediating the antiproliferative activity of Compound 5, cells were incubated with the indicated concentrations of Compound 5, either in the presence or absence of 5 µM U-0126 for 72h. Cells were harvested, stained with Hoechst 33258 and cellular DNA was quantified by fluorimetry as previously described (17). Figure 7 shows that inclusion of
the MEK inhibitor significantly reduced Compound 5-mediated cell growth inhibition. This strongly suggests that activation of the Erk pathway is the major determinant in the antiproliferative effects of Compound 5. In contrast, p38 activation, which has been implicated in cell death in many cell types, did not appear to mediate growth inhibition of Hep3B cells by Compound 5 since in the presence of U-0126, cell growth continued despite high levels of nuclear phospho-p38 but depressed levels of phospho-Erk (see Figure 6).

The effects of Compound 5 on nuclear phospho-Erk accumulation are cell type dependent. To determine whether the observed accumulation of phospho-Erk was specific for Hep3B cells, we examined the ability of Compound 5 to induce nuclear phospho-Erk accumulation in a variety of mammalian cell lines using the ArrayScan II. We found Compound 5-induced nuclear phospho-Erk accumulation was not unique to Hep3B cells but that the magnitude of response varied with cell type. Cell lines fell into three categories based on the magnitude of phospho-Erk induction. Strong responders were NIH 3T3, Rat-1, and Hep3B cells, which showed up to 24-, 75-, and 57-fold increases over control cells, respectively, in nuclear phospho-Erk levels 30 min after exposure to 10 µM Compound 5 (data not shown). DU-145 and PC-3 prostate cancer cells were less responsive (2-3 fold increase with 30 min exposure to 10 µM Compound 5), and HeLa cells did not respond to Compound 5 with enhanced nuclear phospho-Erk accumulation at concentrations up to 30 µM (data not shown). Thus, the induction of phospho-Erk nuclear accumulation by Compound 5 was not limited to Hep3B cells, but instead constituted a more generalized phenomenon.
**Compound 5 restores phospho-Erk levels after Cdc25A overexpression.** Because *in vitro* studies had shown that Compound 5 was most effective against the Cdc25 family of DSPases (12), we investigated whether the effects of a brief treatment with Compound 5 on phospho-Erk nuclear accumulation could be attributed to Cdc25A inhibition. Previous reports have revealed that the tyrosine phosphorylation status and activity of Raf-1, which is an upstream activator of Erk, is controlled by Cdc25A (16). Thus, we hypothesized that ectopic expression of Cdc25A might reduce Erk phosphorylation and provide a novel assay system to examine the acute actions of Compound 5 against intracellular Cdc25A. We selected HeLa cells as a model because in the absence of ectopic Cdc25A no nuclear phospho-Erk accumulation was seen with Compound 5 in these cells, possibly due to low endogenous Cdc25A activity. We predicted that this model would, therefore, have the lowest background and that any effect seen with a small molecule could be assigned to an action on the ectopically expressed Cdc25A. As illustrated in Figure 8A, ectopic Cdc25A expression reduced Erk phosphorylation by 50% (p<0.05, Figure 8B). This reduction in Erk phosphorylation absolutely required the intrinsic phosphatase activity of Cdc25A because a catalytically inactive Cdc25A (C430S) did not reduce Erk phosphorylation in these cells. We then asked whether Compound 5 was able to restore Erk phosphorylation after ectopic expression of wild-type Cdc25A. Cells transiently transfected with wild-type Cdc25A were allowed to recover for 48 h and, during the last 30 min of recovery, treated with vehicle or increasing concentrations of Compound 5. Figure 8B shows that Compound 5 gradually restored Erk phosphorylation to mock/control levels. Consistent with the inherent
unresponsiveness of HeLa cells to Compound 5 (see above), the levels of Erk phosphorylation in untransfected cells were not markedly changed upon Compound 5 treatment (Figure 8A). Furthermore, phospho-Erk levels in Cdc25A-expressing, Compound 5-treated cells never exceeded those in untransfected cells (Figure 8B). These results support the hypothesis that Compound 5 interfered with Cdc25A-mediated dephosphorylation of Erk.
Discussion

PTPases and DSPases play a major role in receptor-mediated signal transduction events. For example, the kinase activities of growth factor receptors are regulated by tyrosine autophosphorylation. Signals initiated at the cell surface are transmitted by a series of cytoplasmic kinases that sequentially phosphorylate each other, eventually leading to activation of members of the MAPK superfamily, namely Erk, p38, and SAPK/JNK, by dual phosphorylation on tyrosine and threonine residues. PTPases dephosphorylate tyrosines and thus inactivate growth factor receptors, whereas the signal at the level of MAPK is attenuated by dephosphorylation of the MAPKs on both tyrosine and threonine by specific MKPs. Recently, the cell cycle phosphatase Cdc25A has been proposed to regulate tyrosine phosphorylation and activity of Raf-1, a key element in the Erk signaling cascade (16).

In contrast to their upstream activating kinases, tyrosine / threonine phosphorylated MAPKs translocate to the nucleus where they phosphorylate and activate their respective protein targets, which include several transcription factors. Very few studies have addressed spatial aspects of phosphorylation-dependent signaling events and to our knowledge, none have investigated small molecules that might perturb the subcellular localization of key signal transducers in the context of tyrosine phosphorylation. Here we have used a high-content, cell-based assay to evaluate the temporal and spatial dynamics of three key parallel signaling molecules in response to Compound 5, a synthetic K vitamin analog with in vitro antiphosphatase activity (12) and antiproliferative activity in a variety of cell lines (10). Using this novel methodology, we found that Compound 5
caused rapid and irreversible nuclear accumulation of phospho-Erk and phospho-p38.

The observed activation of Erk by a compound known to cause growth inhibition (11-12, 17) is somewhat surprising since brief activation of Erk is often associated with mitogenesis and survival. In contrast, JNK and p38 are thought to be mediators of stress responses and apoptosis (24). We considered the possibility that p38 activation might be a factor in the antiproliferative activity of Compound 5. In the presence of the MEK1 inhibitor U-0126, however, which inhibits Erk activation, Hep3B cells grew despite having high levels of phospho-p38. In contrast, we found that pretreatment of cells with U-0126 not only prevented Compound 5-induced Erk phosphorylation, but also protected cells from the growth inhibitory effects of Compound 5. These results strongly support our previous suggestion that prolonged activation of the Erk pathway is causally involved in the growth inhibitory effects of Compound 5 (13, 17). Moreover, our conclusion is in agreement with a growing body of data documenting an involvement of Erk in growth inhibition in neuronal cells (25), NIH3T3 cells (26), and MCF-7 cells (14). In addition, there is increasing evidence that p38 does not appear to exclusively mediate cytotoxicity, but can be cytoprotective under certain conditions (27-29).

The inability of Compound 5 to induce NFkB and, to a lesser extent, JNK, suggests specificity and that it is not a general stress-inducing stimulus. Both NFkB and JNK are activated by a variety of extracellular stimuli, such as oxidative stress or inflammatory cytokines. In addition, the broad PTPase inhibitors vanadate and pervanadate have been found to induce NFkB (30, 31), providing further support for a unique and more specific action associated with Compound 5. We recently demonstrated that Compound 5
selectively inhibited members of the DSPase family with median inhibitory values of 4 µM for Cdc25B₂ and Cdc25A, while it was 10-fold less active against VHR, a prototype MKP, and 100-fold less active against PTP1B (12). Furthermore, Compound 5 caused a cell cycle arrest in both G1 and G2, which correlated with enhanced phosphorylation of the Cdc25 substrates Cdk1, Cdk2, and Cdk4, respectively (12). We suggested that the growth inhibitory properties of Compound 5 might be due to inhibition of the Cdc25 family, but in large part due to a lack of appropriate assays, there has been no direct evidence that Compound 5 inhibits Cdc25 phosphatases in the cell. To investigate whether Cdc25A could affect Erk phosphorylation status and be inhibited within cells by Compound 5, we devised a chemical complementation strategy based on earlier observations that Cdc25A associated with the Raf-1 oncoprotein (15). Functional evidence that Cdc25A regulates Raf-1 activity was obtained by Xia et al. (16), who showed that overexpression of Raf-1 together with wild-type Cdc25A reduced PDGF-mediated Raf-1 tyrosine phosphorylation in NIH 3T3 cells. Raf-1 is one of the most important upstream activators of the Erk cascade (32). We thus hypothesized that Cdc25A overexpression would result in decreased Erk phosphorylation and that an inhibitor of Cdc25A would restore Erk phosphorylation to normal levels, by chemically complementing the loss-of-function phenotype caused by Cdc25A overexpression. To simplify the analysis, we chose HeLa cells, which did not respond to Compound 5 with increased nuclear phospho-Erk accumulation. By treating Cdc25A-overexpressing cells with concentrations of Compound 5 that did not cause Erk hyperphosphorylation under normal growth conditions, we were able to demonstrate that Compound 5 specifically inhibited the effects of the overexpressed Cdc25A protein on Erk phosphorylation. Thus,
we have obtained, for the first time, evidence that Cdc25A regulates endogenous Erk phosphorylation status in whole cells, and that Compound 5 affected Cdc25A function in the cell.

Although the concentrations of Compound 5 required for inhibition of the MKP VHR in vitro are an order of magnitude higher than those for Cdc25A inhibition, it is possible that inhibition of MKPs by Compound 5 also contributes to Erk and p38 activation. A number of cytosolic and nuclear MKPs, which have overlapping substrate specificities, have been described. For example, the Erk isoforms are selectively inhibited by MKP-3, whereas M3/6 selectively dephosphorylates JNK (33). MKP-1 and 2 preferentially dephosphorylate JNK, but also have some activity toward p38 (34, 35). More recently, a p38 specific phosphatase, MKP-5, has been reported (36). The prototype DSPase VHR, which seems to reside in the nucleus, dephosphorylates Erk (37), but its effect on other kinases has not been examined. The fact that Compound 5 only partially activated JNK suggests that it may have some selectivity. At this time, we do not have any information about whether Compound 5 has any specificity for the different MKPs, but this information should become available as we expanding our chemical complementation strategy to probe for cell-active inhibitors of MKPs.

In summary, using the ArrayScan II, we were able to quickly and quantitatively probe selective activation of tyrosine phosphorylation-dependent signal transduction events by a small molecule dual-specificity phosphatase inhibitor in intact cells. By performing fluorescence-based spatial analysis in a high-throughput compatible format, we
demonstrated that this inhibitor selectively activated dual-specificity phosphatase-dependent cellular events. Subsequent analyses using both genetic and pharmacological tools identified activation of the Erk pathway as the dominant component mediating Compound 5’s antiproliferative activity, and provided direct evidence that it could interfere with Cdc25A function in the cell. We propose that the combination of high-content, cell-based analyses coupled with a chemical complementation approach will be a powerful technique to identify cell active inhibitors of a variety of cellular targets.
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Footnotes

1Abbreviations

DSPase, dual-specificity phosphatase; Erk, Extracellular signal-regulated kinase; Hsp90, heat shock protein 90; IL-1α, Interleukin-1 alpha; JNK, c-jun terminal kinase; MAPK, mitogen-activated protein kinase; MKP, mitogen-activated protein kinase phosphatase; NFκB, nuclear factor kappa B; PTPase, protein tyrosine phosphatase; SDS-PAGE, sodium polyacrylamide gel electrophoresis; STPase, serine/threonine phosphatase; VHR, VH-1-related phosphatase.

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Figure Legends

Figure 1. Quantitation of phospho-Erk nuclear accumulation in Compound 5-treated Hep3B cells by the nuclear to cytoplasmic translocation algorithm.

Untreated (A-C) and Compound 5-treated (D-F) Hep3B cells were stained with Hoechst 33342 fluorescent dye (A and D) or with an anti-phospho-Erk antibody followed by a fluorescently tagged secondary antibody (B, C, E, F). Images were acquired in two separate channels on an ArrayScan II system, and analyzed for both nuclear and cytoplasmic phospho-Erk expression. Nuclear masks were generated from Hoechst 33342-stained nuclei, and analysis parameters adjusted to exclude irregularly shaped or sized nuclei as well as aggregate cells. For determination of cytoplasmic intensity, the nuclear boundary was eroded by two pixels, and fitted with two concentric circles placed around the nuclear mask (panels C and F). Cytonuclear differences were calculated by subtracting the average cytosolic fluorescence pixel intensity from the average nuclear fluorescence pixel intensity. Bar = 55 µm.

Figure 2. Selective activation of Erk and p38, but not NFkB by Compound 5.

Hep3B cells (4,000) were plated in each of the 96 wells of a darkwell plate, treated with Compound 5 or vehicle, and stained with anti-phospho-Erk, phospho-p38, phospho-JNK, and p65NFkB antibodies. A minimum of 100 cells per well were analyzed with the previously described (18) nuclear to cytoplasm translocation algorithm on the ArrayScan II (Cellomics, Pittsburgh, PA). Cytoplasm-to-nuclear difference values were calculated
as described in the legend to Figure 1 and normalized to the maximum signal obtained (10 µM Compound 5 for Erk and p38, and 25 ng/ml IL-1α for JNK and NFkB). Data shown are the averages from quadruplicate wells ± S.D. and are from a single experiment that has been repeated at least two times with identical results.

**Figure 3. Kinetics and concentration-dependence of Compound 5-induced phospho-Erk and phospho-p38 nuclear accumulation.** Hep3B cells were incubated for 30 min with increasing concentrations of Compound 5 (A), or for the indicated amounts of time with 10 µM Compound 5 (B). Average cytonuclear differences were obtained by quantitation of phospho-Erk or phospho-p38 staining. Data are the averages ± S.E.M. from quadruplicate wells, with approximately 100 cells being scored in each well.

**Figure 4. Pulse-treatment with Compound 5 and partial activation of Erk and p38.** Cells were treated for various lengths of time with Compound 5 followed by compound removal and incubation in fresh medium. After a total of 30 min, cells were washed, fixed and stained with anti-phospho-Erk (open bars) or anti-phospho-p38 (solid bars) antibodies. Numeric values for phospho-Erk and phospho-p38 nuclear accumulation were obtained as described in the legend to Figure 1, and data were normalized to the maximum signal obtained (30 min of continuous exposure to Compound 5). Data are the averages ± S.E.M. from quadruplicate wells. Similar results were obtained in a second independent experiment.
Figure 5. **Confirmation of Compound 5-mediated phospho-Erk activation by immunoblot analysis and in vitro kinase assay.** Hep3B cells were grown to subconfluency in 100 mm dishes, treated for the indicated lengths of time with Compound 5 (10 µM), and harvested. (A) Nuclear and cytoplasmic fractions of treated and untreated Hep3B cells were separated on SDS-PAGE and immunoblotted with an anti-phospho-Erk antibody (p-Erk). Equal protein loading and the quality of the cellular separation procedure were demonstrated by reprobing the identical blots with anti-Oct-1 (nuclear marker) or anti-Hsp-90 (cytosolic marker) antibodies. (B) Proteins from nuclear and cytosolic fractions were immunoprecipitated with anti-phospho-Erk antibody-agarose conjugate, and the immunoprecipitates subjected to an in vitro kinase assay using recombinant GST-Elk-1 fusion protein. Reaction mixtures were separated on SDS-PAGE and immunoblotted with an anti-phospho-Elk-1 antibody. Data shown are representative of three experiments giving similar results.

Figure 6. **Inhibition of phospho-Erk, but not phospho-p38 or phospho-JNK nuclear translocation by a MEK inhibitor.** Hep3B cells were pretreated with 10 µM U-0126 for 45 min, and subsequently with vehicle (open bars), 10 µM Compound 5 (closed bars) or a mixture of 10 µM Compound 5 and 10 µM U-0126 (hatched bars). After 30 min, cells were fixed and stained with anti-phospho-Erk, anti-phospho-p38, or anti-phospho-JNK antibodies. Data shown are the averages, normalized to the maximum signal obtained, from quadruplicate wells ± S.D. The conditions for maximum stimulation were: 10 µM compound for Erk and p38; 25 ng/ml IL-1α for JNK and NFkB. Results are from a single representative experiment that has been repeated at least two times.
Figure 7. Protection of cells by the MEK inhibitor U-0126 from Compound 5-mediated growth inhibition. Hep3B cells were grown in 12 well tissue culture plates and treated every 24 h with the indicated concentrations of Compound 5 in the presence (closed squares) or absence (open squares) of 5 µM U-0126. After three days, the medium was removed and cell number estimated by fluorimetric quantitation of cellular DNA as described in “Experimental Procedures”. Data are the averages from seven independent experiments, performed in duplicate, ± S.D. ** p < 0.005, *** p < 0.001 as determined by Student’s t-test (two-tailed, assuming unequal variances).

Figure 8. Restoration of Erk phosphorylation in cells overexpressing Cdc25A by Compound 5. Panel A. HeLa cells were transfected with plasmids encoding wild-type or mutant (C430S) Cdc25A and allowed to recover for 48 h. Where indicated, cells were treated with various concentrations of Compound 5 during the last 30 min of recovery, lysed, and analyzed for phospho-Erk levels by Western blot analysis using an anti-phospho-Erk (phospho-p44/42) antibody. Protein bands were quantified by densitometry. p42, total Erk 2 loading control. Panel B shows Erk phosphorylation values in percent of mock-transfected control, averaged from the number of experiments indicated in the bars ± S.E.M. * p < 0.05, ** p < 0.005 as determined by Student’s t-test (two-tailed, assuming unequal variances) compared with mock-transfected control.
Vogt et al., Figure 1

(A) 

(B) 

(C) 

(D) 

(E) 

(F)
Figure 4

Vogt et al., Figure 4

![Bar graph showing avg cytonuc difference (% of maximum) for unstimulated, 5 min pulse, 10 min pulse, and 30 min continuous conditions.](http://www.jbc.org/Downloaded from)
Figure 5

A  
| cytosol | nucleus |
|---------|---------|
| 0 | 1 | 5 | 10 | 30 | 0 | 1 | 5 | 10 | 30 | min |
| p-Erk |
| Oct-1 |
| Hsp90 |

B  
| GST p-Elk |

Vogt et al., Figure 5
Vogt et al., Figure 6

![Bar graph showing avg. cytonuc difference (% of maximum) for Erk, p38, and JNK with different treatments: unstimulated, 10 μM cpd 5, and cpd 5 + U0126.](image-url)
Vogt et al., Figure 7

![Graph showing DNA content (% of control) vs. Compound 5 (µM). The graph displays two lines, one with square markers and the other with square markers. There are error bars and significance levels indicated with asterisks.](http://www.jbc.org/Downloadedfrom)
Vogt et al., Figure 8

Cdc25A transfection: mock, C430S, wild-type, mutant, vehicle. Compound 5 (µM): 0, 5, 10, 20, 5, 10, 20. Phospho-p44, phospho-p42, p42.

Bar graph: mock, wild-type, mutant, vehicle, wild-type, 5µM C5, wild-type, 10µM C5, wild-type, 20µM C5. pErk levels (percent of control): 0, 50, 100.
Spatial analysis of key signaling proteins by high-content solid-phase cytometry in Hep3B cells treated with an inhibitor of Cdc25 dual-specificity phosphatases
Andreas Vogt, Takahito Adachi, Alexander P. Ducruet, Jon Chesebrough, Kaoru Nemoto, Brian I. Carr and John S. Lazo

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