[32] Protein-Fragment Complementation Assays (PCA) in Small GTPase Research and Drug Discovery

By JOHN K. WESTWICK and STEPHEN W. MICHNICK

Abstract

Small GTPases of the Ras and Rho families are among the most studied signaling proteins and represent promising therapeutic targets for human neoplastic disease. Despite the high level of interest in these proteins, direct analysis of most aspects of Ras protein biology in living cells has not been possible, because much of the details of Ras signaling cannot be studied *in vitro* but requires simple cell-based assays. Here we describe a strategy for directly analyzing Ras signaling pathways in living cells using protein-fragment complementation assays (PCA) based on fragments of intensely fluorescent proteins. The assays allow for spatial and temporal analysis of protein complexes including those that form upstream and downstream from Ras proteins, as well as complexes of Ras proteins with regulator and effector proteins. We describe high-throughput quantitative microscopic methods to follow temporal changes in complex subcellular location and quantity (high-content assays). Spatial and temporal changes in response to perturbations (chemical, siRNA, hormones) allow for delineation of Ras signaling networks and a general and high-throughput approach to identify drugs that act directly or indirectly on Ras pathways.

Introduction

Small GTPases of the Ras and Rho families are among the most studied signaling proteins and represent promising therapeutic targets for human neoplastic disease. Much of our knowledge, however, has been derived from *in vitro* analyses or from functional assays reporting on a downstream effect of Ras activity (such as cellular transformation or gene expression). As described below, these approaches are limited because the study of key processes requires intact, multiprotein complexes at particular cellular compartments or surfaces. Study of such processes requires localized measurements of protein activities in intact cells. Because of this lack of a direct assay capability, and because these proteins do not fall into a classically ''drug-able'' target class, these proteins have proven to be difficult targets for drug development. Examples of small molecule lead compounds directly binding to small GTPases have only recently emerged (Gao *et al.*, 2004). Drug discovery efforts to date have focused on upstream enzymatic regulators of Ras pathway activation (e.g., screens for receptor tyrosine kinase inhibitors),

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or on Ras posttranslational modification (e.g., farnesyl transferase inhibitor screens), or on downstream kinase-regulated signaling events (e.g., screens for Raf kinase inhibitors). Identification of probes directly regulating Ras family protein activity would enhance our understanding of this area of biology and possibly lead to identification of novel therapeutic agents.

Several dynamic events control Ras protein activity and downstream signaling. First, prenylation, proteolytic processing, and methylation at the carboxy terminus of Ras-family proteins regulate localization and activity (reviewed in Williams, 2003; Winter-Vann and Casey, 2005). Second, adaptor proteins, guanine nucleotide exchange factors and GTPase activating proteins interact with upstream receptors and in turn interact with Ras proteins, regulating their activity by means of the regulation of localization, formation of specific protein complexes, and GDP/GTP exchange. Third, effector proteins interact with Ras in a nucleotide-dependent manner, leading to regulation of downstream signaling events (such as MAP kinase activation) (Spoerner *et al.*, 2001). Therefore, the number, composition, and subcellular localization of Ras-containing protein complexes changes depending on the level of pathway activity. The ability to monitor the localization of Ras and Ras-pathway components is clearly desirable. Given the combinatorial complexity of Ras interactions with cellular structures, activators, and effectors, the added ability to quantify specific complexes would significantly enhance our understanding of these pathways. We describe here a strategy for directly analyzing these events using live cell, high-content protein-fragment complementation assays (PCA). These assays are also amenable to analysis of various events upstream and downstream from Ras proteins and are not limited to a particular target class. Therefore, this strategy may be broadly applicable to analysis of Ras signaling networks.

Methods

Background and General Considerations

Principles and basic methods for PCA have been extensively reviewed elsewhere (Campbell-Valois and Michnick, 2005; Michnick *et al.*, 2000; Remy *et al.*, 2001). PCA involves the use of a rationally dissected reporter protein (including enzymes and fluorescent or luminescent proteins). For the purposes of this review, we will focus on assays that use fragments of inherently fluorescent proteins. The ends of each reporter cDNA fragment are separately fused, in-frame and with a short flexible linker, to two test proteins that are known (or suspected) to interact. The resultant two cDNA fusion expression cassettes are cointroduced into cells. After expression, if the two test proteins interact, the fragments of the reporter

protein are brought within close proximity and spontaneously refold to generate a measurable signal. With fluorescence PCAs, at least three types of events can be observed and quantified—an increase or a decrease in protein complex formation and/or a change in subcellular localization or concentration of the signal. As with full-length fluorescent proteins, one of the key advantages of this strategy is that the location of signals (and thus their cognate protein complexes) can be determined with high precision. For example, signal localization to organelles, suborganelle structures such as nucleoli, and structures as small as clathrin-coated pits can be easily resolved and quantified in high throughput. The abundance of functional dyes for various subcellular structures, coupled with image analysis and deconvolution of multiple wavelength signals, enables colocalization studies. In addition, protein complexes visualized by PCA are not simply binary but can contain other protein and nonprotein components that make up the native multimolecular complexes.

As with any technique involving expression of exogenous proteins, it is important to consider the level of expression. Expression at levels significantly higher than the endogenous protein can abrogate normal regulatory mechanisms within a pathway and for some proteins may be toxic. Some strategies for protein complex analysis, such as epitope-tag affinity-based approaches or techniques involving energy transfer between fluorophores or fluorophores and luminescent enzymes (FRET, BRET) require high levels of exogenous protein expression. We have found, using intensely fluorescent variants of YFP for PCA engineering, that expressing proteins at or below the endogenous level can still yield readily quantifiable signals (Yu *et al.*, 2004).

PCA has been used in a number of common cell lines, including CHO, HEK293, HeLa, Cos, U2OS, Hep3B, HepG2, and Jurkat, as well as insect and plant cells and bacteria (Lamerdin and Westwick, unpublished; Leveson-Gower *et al.*, 2004; Nyfeler *et al.*, 2005; Pelletier *et al.*, 1998; Remy and Michnick, 2004b; Remy *et al.*, 2004; Subramaniam *et al.*, 2001). Recent extension of these assays into viral vector systems suggests that any transduceable cell type, including nondividing cells and stem cells, can be analyzed with this strategy. We describe in the following the method for transient PCA expression in HEK cells with concomitant drug or siRNA treatment.

Fragment Synthesis and Construct Preparation

Fusion constructs using cDNAs coding for full-length proteins are generated as described previously (Yu *et al.*, 2004) or with YFP reporter fragments with the following additional mutations: YFP[1]-(F46L, F64L, M153T) and YFP[2]-(V163A, S175G). These mutations have been shown to enhance chromophore maturation and increase the fluorescence intensity

of the intact YFP protein (Nagai *et al.*, 2002). Each test cDNA can be fused to the reporter fragment at either the amino or carboxy terminus. In addition, either fragment of the reporter can be used for each test protein, yielding four possible fusion constructs for each protein and eight possible combinations between two interacting proteins. In practice, all possible combinations should be tested for a pair of interacting proteins. Pairs yielding sufficient signal and correct subcellular localization are chosen for further analysis.

Cells and Transfections

HEK293 cells are maintained in MEM alpha medium (Invitrogen) supplemented with 10% FBS (Gemini Bio-Products), 1% penicillin, and 1% streptomycin, and grown in a 37° humidified incubator equilibrated to 5% CO_2. Cells are seeded at 7500 cells per well in 96-well plates 20 h before transfection and cotransfected with up to 100 ng total of complementary fusion vectors using Fugene 6 (Roche) according to the manufacturer's protocol. The amount of each vector used needs to be empirically determined to identify the lowest DNA concentration at which a quantifiable signal is obtained. We have found that transient transfection of many PCAs generates quantifiable signals in the appropriate cellular compartments that respond appropriately to pathway stimulation or inhibition. Predictably, many PCAs generate a more homogeneous signal when engineered as stably transfected cell lines. For high-throughput screening campaigns, engineering of a clonal cell line is, therefore, advisable.

Image Acquisition and Analysis

These assays are amenable to imaging on any platform capable of acquiring fluorescence signals, including microscopes, flow cytometers, and simple plate readers. We focus here on the use of confocal or epifluorescence microscopy systems and recently developed high-throughput derivatives. We have used the Discovery-1 automated fluorescence imager (Molecular Devices, Inc.) equipped with a robotic arm (CRS Catalyst Express; Thermo Electron Corp., Waltham, Mass), as well as the Opera high-throughput confocal fluorescence imaging platform (Evotec Technologies, Hamburg). For the Discovery-1, the following filter sets are used to obtain images: excitation filter 480 ± 40 nm, emission filter 535 ± 50 nm (YFP); excitation filter 360 ± 40 nm, emission filter 465 ± 30 nm (Hoechst); excitation filter 560 ± 50 nm, emission filter 650 ± 40 nm (Texas Red). A constant exposure time for each wavelength is used to acquire all images for a given assay. For higher throughput analyses, cells are fixed and stained 48 h after

transfection as described previously. However, live cell imaging is possible, enabling real-time tracking and ''movies'' of signaling dynamics.

Raw images in 16-bit gray scale TIFF format are analyzed using modules from the ImageJ API/library (http://rsb.info.nih.gov/ij/, NIH, MD). First, images from each fluorescence channel (Hoechst, YFP, and Texas Red) are normalized using the ImageJ built-in rolling-ball algorithm (Sternberg, 1983). Because each PCA generates signal in a specific subcellular compartment or organelle, and treatment with a drug or siRNA may effect a change in complex localization or signal intensity, different algorithms are required to accurately quantitate fluorescent signals localized to the membrane, nucleus, or cytosol. Each assay was categorized according to the subcellular localization of the fluorescent signal, and changes in signal intensity across each sample population were quantified using one of multiple automated image analysis algorithms. A detailed description of specific algorithms is beyond the scope of this review, but it should be noted that, as with most cell-based assays, the signals across the population do not form a normal distribution. We and others have found that the nonparametric statistical approaches to these data have proven to be the most useful (Giuliano *et al*., 2003).

Applications and Examples

Mapping Signaling Complexes, Pathways, and Networks

PCA has been used extensively to identify novel interacting partners for known signaling proteins and to map signaling networks. For example, we have devised a PCA-based system to screen cDNA libraries to identify novel proteins implicated in signaling by the protein kinase PKB (Remy and Michnick, 2004a,b; Remy *et al*., 2004). It should be noted that these studies map protein complexes, not just binary protein interactions, because a positive signal is not necessarily dependent on a direct interaction between two proteins. If proteins are in sufficiently close proximity in the context of a larger protein complex, a positive signal can result. The absolute proximity of two proteins required to generate a signal is a function of their structure and the length of polypeptide linkers seperating proteins and complementary fragments (Remy *et al*., 1999), but it should be noted that a positive PCA signal is essentially an ''all-or-none'' phenomenon. That is, PCA results from *folding*, not association, of the reporter protein fragments. Protein folding is a highly cooperative process in which, once conditions are created for a polypeptide to fold (in the case of PCA, when the fragments are brought together by the interacting proteins), folding will proceed to completion spontaneously. A further consequence of this

all-or-none behavior is the extremely high dynamic range of PCA compared with FRET-based interaction assays, regardless of the relative expression levels of the proteins of interest (Michnick, 2001; Zhang *et al.*, 2002).

Figure 1 provides examples of the wide range of small GTPase-related signaling events that can be probed with PCA. Diverse processes and target classes can be visualized and quantified, including upstream activators of Ras protein regulation (integrin dimerization; ITGα5/ITGβ1), GTPase/scaffold interactions (Cdc42/WASP), guanine nucleotide exchange factor/GTPase complexes (Vav/Cdc42), GTPase/effector complexes (Cdc42/Pak4), and downstream signaling events (Raf, MEK, Elk, and SRF-containing protein complexes; Fig. 1). Also notable is the fact that multiple interactions involving the same protein can be probed, for example, complexes of Raf with

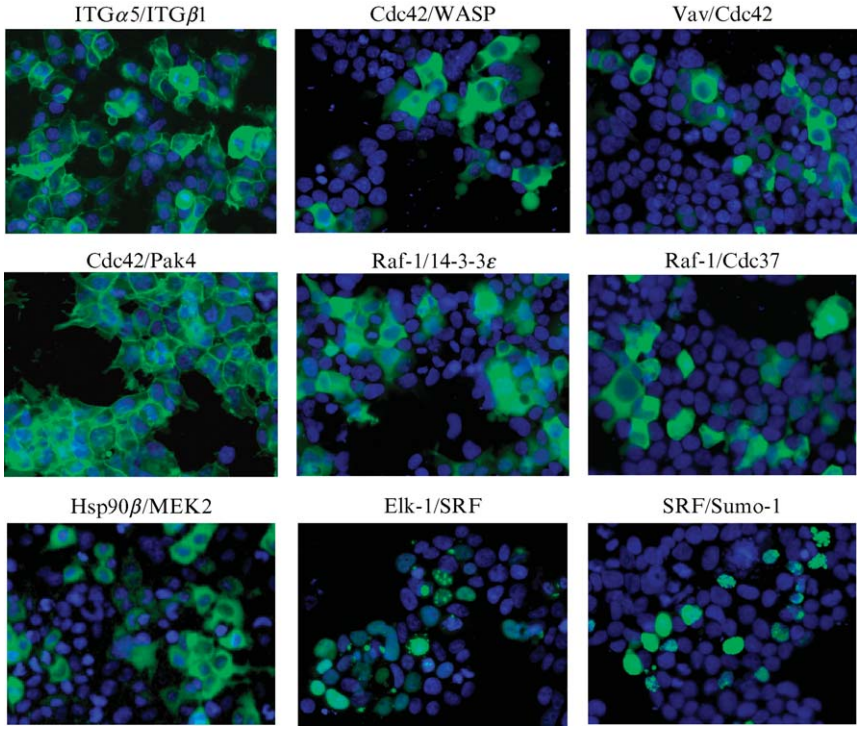

FIG. 1. Examples of GTPase-related signaling activities probed with PCA. HEK293 cells were transfected with the indicated pairs of PCA vectors; 48 h after transfection, cells were fixed and stained with Hoechst, and images were captured on the Discovery 1 as described.

modulatory proteins or chaperones (14-3-3 or Cdc37; Fig. 1) or downstream effectors (Raf/Mek; see Fig. 3).

These examples span multiple steps in Ras-related signaling pathways and probe signaling events specific to distinct subcellular compartments. For example, a major fraction of ERK protein kinase pools resides in the cytoplasm of these cells in a complex with its activators MEK1 and MEK2 (Fig. 3 and data not shown), but by using an assay such as ERK/ELK or ELK/SRF, events specific for nuclear ERK can be visualized and quantified (Fig. 1). Importantly, the response of the common protein to drugs or siRNAs is often dependent on the specific context in which it is probed.

Probing Pathway Architecture and Signaling Dynamics

A unique feature of PCA compared with other interaction mapping strategies such as yeast two-hybrid analysis, phage display, affinity purification, or other proteomics-based approaches is the ability to capture the dynamics of complexes in their native context. In conjunction with high content cell imaging, detailed information on the quantities and localization of signaling complexes is obtained. Thus, the assays can be used not just to identify the components of signaling networks but also to identify the activity of pathways within these networks. The examples described in the following demonstrate that it is possible to probe discrete signaling nodes for agents that act directly on the signaling proteins of which it is composed, as well as for targets and agents that act ''upstream'' of the node. The nodes are PCA-detected protein–protein interactions that report on specific steps in a signal tansduction cascade analogous to transcriptional reporter genes, except that analysis covers multiple events in a pathway (from membrane to nucleus) rather than being limited to an endpoint transcriptional response to pathway modulation.

Because PCA assays are generally performed as plasmid-based transfections, the strategy works well in conjunction with other genetic probes requiring transfection, such as plasmids encoding dominant negative/active proteins and RNAi. For example, the JNK2/c-Jun PCA was cotransfected with a constitutively active signaling protein, pDCR.RasV12 (White *et al.*, 1995) or the corresponding empty vector (pDCR) (Fig. 2). A dramatically higher level of fluorescence was seen in cells cotransfected with only 1 ng of Ras cDNA (activated G12V mutant). The JNK-Jun pathway is known to respond to Ras activation (Westwick *et al.*, 1994), and this response was robust, occurring over a range (30–100 ng) of PCA vector transfection levels (Fig. 2).

PCA and siRNA strategies are highly complementary. The cotransfection frequency—determined with fluorescently labeled siRNAs cotransfected with fluorescence PCAs—approaches 100% (data not shown). For

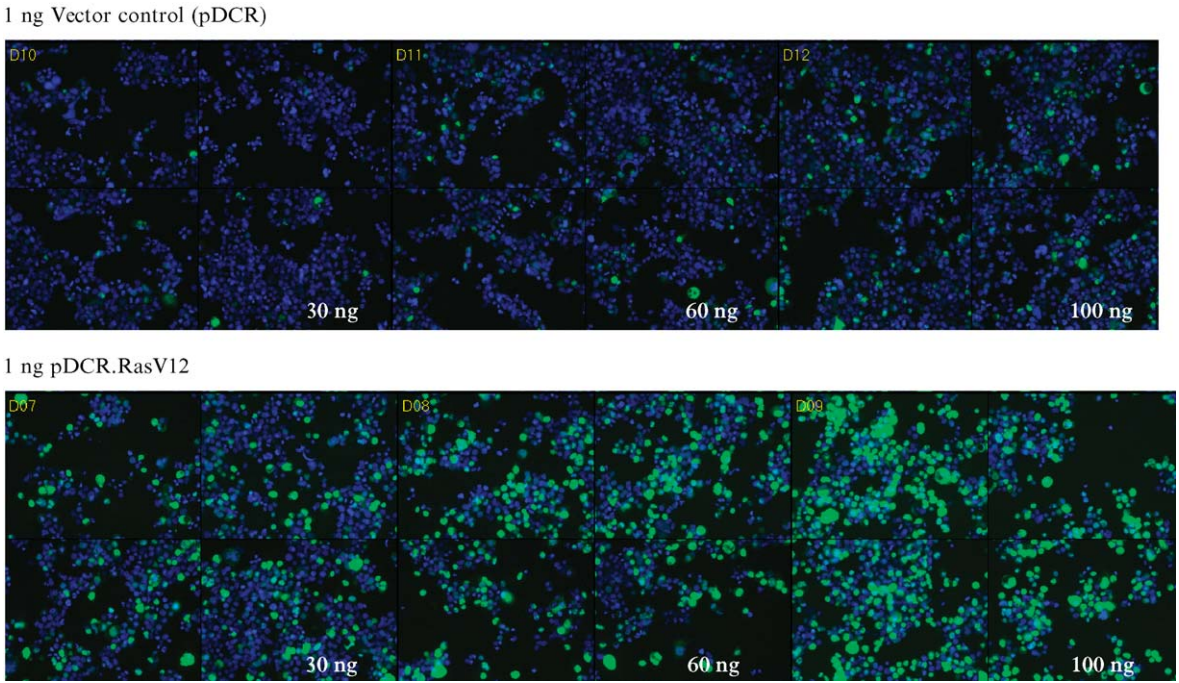

FIG. 2. Activation of a JNK2/c–Jun signaling complexes after cotransfection of Ras (G12V). HEK 293 cells were transfected with JNK2 and c-Jun PCA fusion vectors (30, 60, or 100 ng total PCA vector DNA, as indicated) along with 1 ng of empty vector (pDCR) or 1 ng of PDCR Ras G12V. Cells were fixed, stained, and imaged as described in the text.

assessment of siRNA-mediated target knock-down, siRNAs are transiently cotransfected with PCA plasmid vectors pairs. Lipofectamine 2000 is the optimal transfection reagent for siRNA/PCA cotransfections. siRNA SMART pools designed to target human genes and two ''GC-matched'' nonspecific siRNA pools were designed and obtained from Dharmacon (Boulder, CO). siRNA pools directly targeting one of the components of a PCA used in the study serve as a control for siRNA efficacy (such as H-Ras siRNA and the H-Ras/Raf PCA; Fig. 3). siRNA pools are generally designed to target endogenous proteins, allowing analyses of the effects of endogenous protein knockdown on pathway activity. To determine the optimal siRNA concentration at which targets are modulated but nonspecific effects are minimized, we evaluated the effects of siGFP (Dharmacon) and the nonspecific siRNA controls on four different PCAs. Under the conditions listed here, 40 n*M* siRNA was found to be optimal. Although higher concentrations (100 n*M*) are typically reported and lead to higher levels of target protein knockdown, we found evidence of widespread nonspecific activity at these concentrations (data not shown). siRNA effects on PCA activity are quantified as described previously and compared with the pooled mean fluorescence of the corresponding nonspecific siRNAs. Each 96-well plate should contain five internal controls: mock (no PCA), no siRNA, nonspecific siRNA controls (such as control IX and control XI; 47% and 36% GC content, respectively, Dharmacon, Boulder, CO), and a PCA-specific control (to confirm degree of stimulation for assays treated with agonists).

Figure 3 illustrates several PCAs that were cotransfected with a siRNA pool targeting H-Ras or a GC-matched siRNA control. As expected, H-Ras siRNA knocked down complexes of H-Ras with Raf, Raf with MEK1, and MEK1 with ERK2. It is notable that the Ras/Raf complexes are distinctly localized at the plasma membrane (Fig. 3, top left image), whereas the Raf/MEK and MEK/ERK complexes are localized in the cytoplasm. Raf proteins associate with the effector domain of active Ras proteins, an event known to occur at the plasma membrane (Stokoe *et al.*, 1994). By probing the Ras/Raf complex (as opposed to, for example, fluorescently labeled Raf protein alone), this assay focuses exclusively on active signaling complexes. In addition, although MEK/ERK complexes were seen at the cell membrane and in the cytoplasm, complexes of ERK with transcription factor substrates or complexes of these substrates with other proteins (such as ELK/SRF complexes) are seen exclusively in the nucleus (Fig. 1, and data not shown). Thus, the combination of complex-based assays and high content image analysis can yield information on specific steps in a pathway, not just the endpoint.

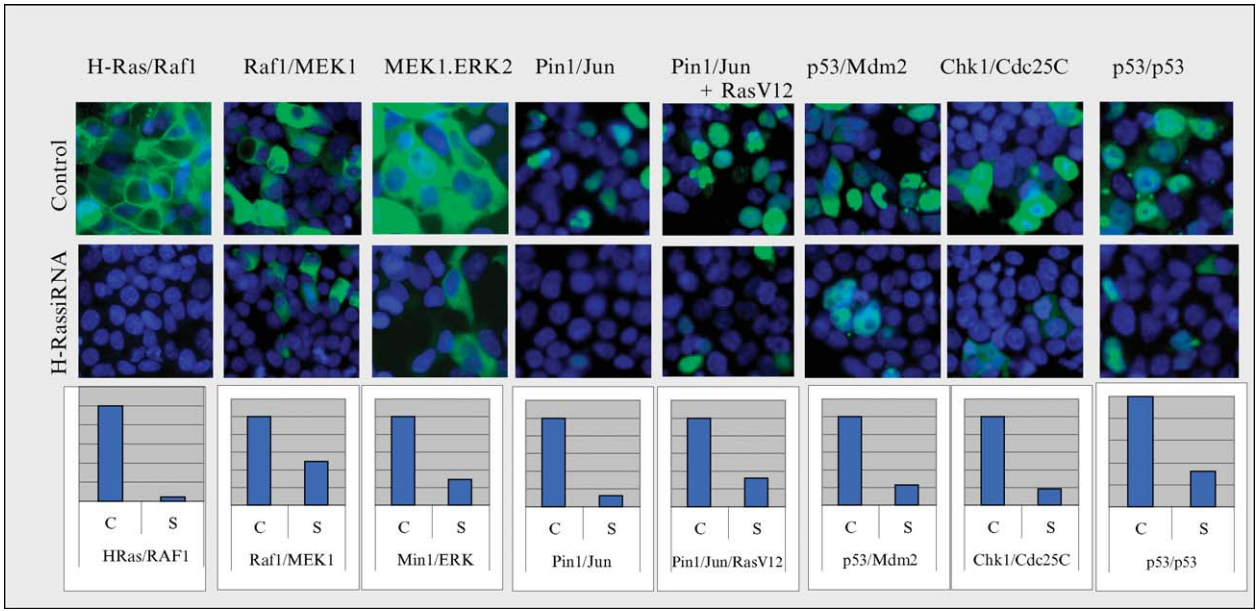

FIG. 3. siRNA-mediated Ras knockdown reveals downstream signaling connections. The indicated PCA pairs were cotransfected with 40 ng of control GC-matched siRNA or a siRNA pool targeting H-Ras, as indicated; 48 h after transfection, cells were fixed and stained as described in the text and imaged on the Discovery 1. Image analysis was performed as described in the text, and results are shown below the corresponding images. C, control siRNA pool; S, H-Ras siRNA pool.

Other events known to be downstream of Ras activation are inhibited after cell treatment with Ras siRNA. For example, c-Jun is phosphorylated and activated (and hence complexes with the prolyl isomerase Pin1) after activation of the Ras pathway (Wulf *et al.*, 2001). In logarithmically growing cells, an appreciable level of Pin1/Jun complexes is evident and exclusively localized in the nucleus, as expected (Fig. 3). If an activated allele of H-Ras (Ras V12) is cotransfected with the PCA pair, the level of nuclear Pin1/Jun complexes increases dramatically and also adopts a distinct pattern, suggesting enhanced interaction with chromatin. In each case, cotransfection of siRNA targeting H-Ras clearly diminishes the levels of Pin1/Jun complexes, indicating that endogenous H-Ras is involved in the pathway leading to formation or stability of these complexes. Several PCAs in the cell cycle control/DNA damage response paths were also regulated by treatment with H-Ras siRNA, indicating that Ras controls pathways leading to these protein complexes (p53/Mdm2, Chk1/Cdc25C, and p53/p53; Fig. 3). Thus, both expected and unexpected connections between pathways can be visualized and quantified with this strategy.

HTS and Probing Novel Targets

Dramatic improvements in high-content screening instrumentation, image analysis algorithms, and data mining tools have ushered in an era where these types of strategies are realistic choices not only for secondary screening and mechanism of action studies but primary high-throughput screening as well (Giuliano *et al.*, 2003). Although instrumentation and analysis tools have improved dramatically, the scope of assays available for use on these platforms has remained limited. The strategy described here is not limited to traditional measures of protein level or posttranslational modification and, therefore, adds extensively to the list of potential assays for pathway mapping and therapeutic discovery. In addition to enabling direct probing of small GTPase signaling complexes (such as Cdc42, Fig. 1, and H-Ras, Fig. 3), we demonstrate a diverse range of protein targets that can be probed with this approach. Assays representing a wide range of target classes have been constructed, including kinase/kinase complexes (e.g., Raf/MEK and MEK/ERK; Fig. 3), GTPase/scaffold complexes (e.g., Cdc42/WASP; Fig. 1), complexes with modulatory proteins (e.g., Raf/14–3-3), and complexes with chaperones and co-chaperones (e.g., HSP90/MEK and Raf/Cdc37; Fig. 1).

In addition to traditional target classes, novel events can be probed. For example, the direct ubiquitination or sumoylation of specific target proteins can be visualized and quantified (e.g., SRF/SUMO; Fig. 1, and data not shown). Other events related to proteasome-mediated regulation can also be probed, such as the interaction of E3 ubiquitin ligases with their client proteins (Mdm2/p53; Fig. 3). Other enzyme/substrate interactions,

such as the prolyl isomerase PIN2 interaction with its substrate c-Jun, provide a unique assessment of this pathway (Fig. 3). Finally, receptor-mediated events, including GPCR, receptor tyrosine kinase, and integrin signaling, are amenable to analysis (e.g., ITGα5/ITGβ1; Fig. 1).

For drug studies in transiently transfected cells, compounds or vehicle controls are generally added 24 or 48 h after transfection, and cells are incubated for various periods of time before imaging. Drug-induced changes in protein complexes have been visualized and quantified within seconds of treatment and can be followed over the course of several days. After drug or other treatments, cells can be stained with 33 μg/ml Hoechst 33342 (Molecular Probes) and 15 μg/ml Texas Red–conjugated wheat germ agglutinin (WGA; Molecular Probes) to localize nuclei and plasma membrane, respectively. Cells can be imaged live or fixed with 2% formaldehyde (Ted Pella) for 10 min. When fixed, cells are subsequently rinsed with HBSS (Invitrogen) and maintained in the same buffer during image acquisition. With the instrumentation and liquid handling capabilities described here, PCAs have been used to screen 20,000 compounds per day.

Pharmacological Profiling

The diversity of the assays described here, and the richness of data provided by high content imaging, provides a unique opportunity for gaining a better understanding of the activity of drugs and drug targets within living human cells. We have found that the combination of a broad panel of assays and equally broad panel of siRNAs and drugs can yield novel information regarding pathway architecture and drug mechanism of action and safety. Challenges include the need for expensive imaging platforms, high throughput liquid handling robotics, and extensive computational capabilities. For example, profiling studies involving hundreds of assays and dozens of drugs routinely require terabytes of image storage capacity. Because of these instrumentation and computational requirements, and the necessity for engineering a broad panel of diverse assays, broad target and drug profiling is currently beyond the scope of most academic laboratories. However, focused use of these tools is achievable using generally available laboratory equipment (such as fluorescence microscopes and plate readers). In addition, the existence of high-throughput, high-content imaging platforms seems likely to become widespread at industrial screening sites and in larger university core laboratories.

Conclusions

There is a growing appreciation for the fact that signaling events occur in the context of large, multiprotein complexes. Changes in protein

complex levels and localization in response to target modulation or drug treatment reflect the activity of the pathways in which these complexes reside. Despite this knowledge of signal transduction mechanics, most of our understanding of pathway architecture and activity is based on measurable transcriptional or posttranscriptional changes or from proteomic approaches using preparations from disrupted cells. Application of strategies such as those described here should improve our knowledge of how these pathways operate in living cells and may provide novel approaches for the identification of therapeutic agents.

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[33] Ras Up-Regulation of Cyclooxygenase-2

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Abstract

Oncogenic mutations in *Ras* (H-*Ras*, N-*Ras*, and K-*Ras*) are found in a wide variety of human malignancies, including adenocarcinomas of the colon, where K-*Ras* mutations often occur early in tumor development and strongly correlate with the transition to invasive adenocarcinoma.

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