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Measuring Drug Action in the Cellular Context Using Protein-Fragment Complementation Assays

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Abstract: Cellular signal transduction occurs in the context of dynamic multiprotein complexes in highly ramified pathways. These complexes in turn interact with the cytoskeleton, protein scaffolds, membranes, lipid rafts, and specific subcellular organelles, contributing to the exquisitely tight regulation of their localization and activity. However, these realities of drug target biology are not addressed by currently available drug discovery platforms. In this article, we describe the use of protein-fragment complementation assays (PCAs) to assess drugs and drug targets in the context of their native environment. The PCA process allows for the detection of protein–protein complexes following the expression of full-length mammalian genes linked in-frame to polypeptide fragments of rationally dissected reporter genes. If cellular activity causes the association of two proteins linked to complementary reporter fragments, the interaction of the proteins of interest enables refolding of the fragments, which can then generate a quantifiable signal. Using a PCA based on a yellow fluorescent protein, we demonstrate that functional (p50/p65) complexes of the heterodimeric nuclear factor-κB transcription factor, as well as the transcription factor subunit p65 and its modulator IκBα, can be visualized and monitored in live cells. We observed similar responses of the PCA assays to the activities of the cognate endogenous proteins, including modulation by known agonists and antagonists. A proof-of-concept high throughput screen was carried out using the p50/p65 cell line, and potent inhibitors of this pathway were identified. These assays record the dynamic activity of signaling pathways in living cells and in real time, and validate the utility of PCA as a novel approach to drug discovery.

Introduction

CELLULAR SIGNALS propagate via the orchestrated interaction of a complex network of proteins consisting of enzymes, their regulators, and structural components. In most cases that have been examined, proteins function as components of multiprotein complexes, and the activity of the complex is determined by its subunit composition. However, the composition of most signaling complexes is not known. Not surprisingly, lead compounds identified by *in vitro* assays using purified target proteins rarely produce the same efficacy when examined in the context of living cells. An appealing alternative strategy would be to directly examine protein activities in the context of living cells. Here we describe the development of protein-fragment complementation assays (PCAs) as such a tool for high throughput drug discovery and lead profiling. PCAs are created by expressing mammalian genes linked in-frame to fragments of rationally dissected reporter genes. The association of two proteins of interest brings together complementary reporter fragments and enables productive folding of the fragments into an active structure (Fig. 1A). The resulting quantifiable signals can be spatially localized in liv-

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ABBREVIATIONS: ALLN, *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal; ERK, extracellular signal-regulated kinase; EYFP, enhanced yellow fluorescent protein; GFP, green fluorescent protein; IKK, IκB kinase; MEK, mitogen-activated protein kinase kinase; N:C, nuclear/cytoplasmic; NFκB, nuclear factor-κB; PBS, phosphate-buffered saline; PCA, protein-fragment complementation assay; TNFα, tumor necrosis factor; YFP, yellow fluorescent protein.

ing cells by automated microscopy.[1,2] Here we report the development of two PCAs and their application to screening compound libraries for modulators of the tumor necrosis factor-α (TNFα)/nuclear factor-κB(NFκB) signaling process.

TNFα is a key immune and inflammatory mediator, and has been implicated in human disease states such as cancer, arthritis, inflammatory bowel disease, cardiovascular disease, asthma, sepsis, and diabetes.[3–9] Transient ligation of cell-surface TNFα receptors leads to profound changes in gene expression that can permanently alter cell fate and phenotype. One of the major effectors of TNFα signals is the transcription factor NFκB.[10,11] The TNFα/NFκB signal transduction cascade can be summarized as follows (Fig. 1B). Binding of TNFα to type I TNFα receptor induces homotrimerization of the receptors.[12] Association of adaptor proteins such as TRAF and TRADD with activated receptors rapidly induces the formation of a large, multisubunit complex containing IκB kinases (IKKs).[13,14] The activated IKK complex phosphorylates IκBα on serine residues 32 and 36 and targets the protein for degradation by the ubiquitin-proteasome pathway.[15] Proteolysis of IκB frees bound NFκB transcription factors and unmasks their nuclear localization signals, leading to translocation of NFκB proteins into the nucleus and transcriptional regulation of target genes. To identify innovative therapeutics for diseases with aberrant NFκB activity, we wished to design assays probing multiple steps of the signal transduction pathway.

We designed yellow fluorescent protein (YFP) PCAs to examine the dynamics of protein complexes in the TNFα/NFκB signaling cascade. Stable cell lines were engineered for two key steps in NFκB activation: p50/p65 and IκBα/p65. We demonstrate that PCA can be used to measure quantitatively both existence and localization of distinct protein complexes in living cells. As a drug discovery strategy, this approach has several advantages. By probing the pathway, rather than isolated components, no judgments as to the optimal target in the pathway need to be made. Moreover, the YFP PCAs enable direct visualization of distinct protein complexes in living human cells in real time, and do not require laborious methods associated with antibody staining in fixed cells.[16] Finally, we validated the p50/p65 and IκBα/p65 PCAs as high throughput screens for inhibitors of the NFκB pathway.

Materials and Methods

DNA constructs

The PCAs described here were first created by introducing the enhanced YFP (EYFP)-specific mutations S65G, S72A, and T203Y[17] into existing oligonucleotide fragments enhanced green fluorescent protein (of EGFP), resulting in fragments designated YFP[1] and YFP[2] corresponding to amino acids 1–158 and 159–239 of the full-length EYFP.[18] Subsequently, PCA vectors were constructed by starting directly with synthetic oligonucleotides corresponding to YFP[1] and YFP[2] (Blue Heron, Inc.). Open reading frames of full-length murine p65, rat mitogen-activated protein kinase kinase-1 (MEK1), human p50, IκBα, and extracellular signal-regulated kinase-1 (ERK1) (mitogen-activated protein kinase-1) were fused in-frame to complementary YFP fragments. The coding regions of p50 and p65 were ligated to the 3′ ends of YFP[1] and YFP[2], respectively, to generate C-terminal fusions. ERK1 was ligated to the 5′ end of YFP[1], whereas IκBα and MEK1 were appended to Y[2] in N-terminal fusions. The fusion genes were subcloned into pCDNA3.1 expression vectors (Invitrogen) with Zeocin selectable marker for YFP[1]-p50, IκBα-YFP[1], and ERK1-YFP[1] and hygromycin marker for YFP[2]- and ERK1-YFP[2]. A linker consisting of $(Gly_4Ser)_2$ separated the genes of interest and the YFP fragments. The flexible linker facilitates complementation of the dissected reporter fragments when they are brought into close proximity by the linked interacting proteins.

Transfection and stable cell lines

HEK293T cells were grown in minimum essential medium Eagle (α modification) (Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bio-Products), 1% penicillin, and 1% streptomycin and maintained in a 37°C incubator at 5% CO_2. Cells were transfected with the p65 YFP[2]-encoding vector, and stable cell lines were selected using 100 μg/ml Hygromycin B (Invitrogen). Selected cell lines were transfected with YFP[1]-p50 or IκBα-YFP[1]. Stable cell lines expressing YFP[1]-p50/YFP[2]-p65 and IκBα-YFP[1]/YFP[2]-p65 were isolated following double antibiotic selection with 50 μg/ml Hygromycin B and 500 μg/ml Zeocin. MEK1-YFP[2]/ERK1-YFP[1] cell lines were derived from simultaneous transfection of the fusion constructs and selection with Hygromycin and Zeocin (Invitrogen). Fluorescence of these lines is stable over at least 25 passages (data not shown). Fugene 6 (Roche) was used for all the transfections according to the manufacturer's directions.

Immunoblot analysis

Parental and engineered cells were seeded at 1×10^6 cells/well in six-well plates. Twenty hours later, these cultures were treated with 50 ng/ml rhTNFα (Roche) for 30 min. Control cultures were left untreated. Cell lysates were harvested by washing each culture dish with cold phosphate-buffered saline (PBS) and then adding 1 ml of lysis buffer[19] at 4°C. Cell lysates were scraped off the dishes, collected in Eppendorf tubes, set on ice for 30 min, and centrifuged at 15,000 rpm for 10 min. Supernatants were transferred to new tubes, and protein concentrations were determined by the Bradford assay (Bio-

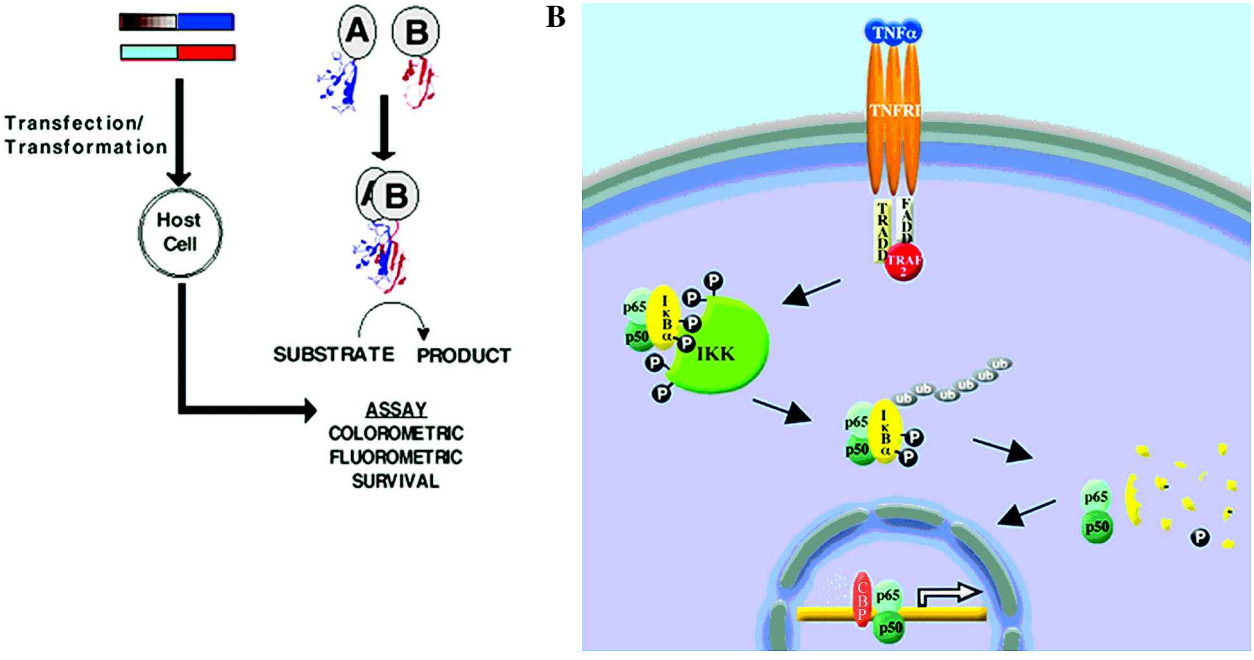


FIG. 1. (**A**) Schematic representation of a PCA. Host cells are transfected with full-length genes fused in-frame to fragments of a rationally dissected reporter enzyme or fluorescent protein (fragments indicated in red and blue). Interaction of the expressed proteins ("A" and "B" in the diagram) results in the productive folding of reporter fragments to generate a functional protein that produces a quantifiable signal. (**B**) TNFα activation of NFκB. TNFα ligation of cell-surface TNFRI triggers recruitment of adaptor molecules TRADD, FADD, and TRAF2, which leads to activation of the IκB kinase (IKK) complex. Phosphorylation of IκBα results in its proteolysis and releases bound NFκB p50/p65 heterodimers. Free NFκB translocates into the nucleus and associates with proteins such as the coactivator CBP to regulate transcription of target genes.

Rad). For immunoblotting, lysates were normalized for protein concentration and combined with NuPAGE 4× LDS Sample Buffer and NuPAGE 10× Sample Reducing Agent (Invitrogen). Proteins were separated in NuPAGE 4–12%, 1.0 mm Bis-Tris gels, and electroblotted onto Invitrolon 0.2-μm polyvinylidene difluoride membranes (Invitrogen). Membranes were blocked with Tris-buffered saline + 0.5% Tween-20 + 5% nonfat dry milk for 40 min, then incubated with mouse anti-NFκB p65 (BD Biosciences), mouse anti-β-actin (Abcam), rabbit anti-green fluorescent protein (anti-GFP; Abcam), or rabbit anti-IκBα (Santa Cruz Biotechnology) for 3 h at room temperature. The membranes were washed with Tris-buffered saline + 0.5% Tween-20 and incubated with horseradish peroxidase-conjugated donkey anti-rabbit antibody or sheep anti-mouse (Amersham), as appropriate. Bands were detected by chemiluminescence using the ECL-PLUS kit (Amersham).

Immunofluorescence

HEK293T cells stably expressing YFP[1]-p50/YFP[2]-p65 were seeded on fibronectin-coated coverslips in a 12-well plate at a density of 1×10^5 cells/well. After 24 h, cells were incubated in the absence or presence of 50 ng/ml TNFα for 30 min. The cells were fixed with 4% formaldehyde in PBS for 15 min, permeabilized by immersion in methanol at −20°C for 20 s, and blocked with 3% bovine serum albumin. Immunostaining was performed using rabbit anti-p65 antibody (Santa Cruz Biotechnology) followed by Cy3-goat anti-rabbit antibody (Molecular Probes). The cells were labeled with nuclear stain Hoechst 33342 at 33 μg/ml for 5 min, mounted on slides, and visualized with a Nikon Eclipse 2000 fluorescence microscope.

Fluorescent PCAs

Cells expressing YFP[1]-p50/YFP[2]-p65, IκBα-YFP [1]/YFP[2]-, or MEK-YFP[2]/ERK1-YFP[1] were seeded at 20,000 cells/well in a black-walled poly-D-lysine-coated 96-well plate (Greiner). Twenty-four hours later, the cells were incubated with human TNFα (Roche) for 30 min. Nuclei were stained with Hoechst 33342 (Molecular Probes) at 33 μg/ml for 10 min. Cells were rinsed with Hanks' balanced salt solution (Invitrogen) and kept in the same buffer. Fluorescence was visualized, and images were acquired using a Discovery-1 automated fluorescence imager (Molecular Devices, Inc.) equipped with excitation and emission filters 470/35 and 535/60, respectively. Where indicated, cells were treated with 25 μM *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN; Calbiochem) for 60 min and induced with TNFα in the continued presence of the inhibitor. For HTS, cells were

pretreated with compounds (10 μM) for 60 minutes and then stimulated with TNFα for 30 min in the presence of drugs. Cells were then fixed with 2% formaldehyde in Hanks' balanced salt solution and subsequently stained with Hoechst 33342. All liquid handling was done using a Biomek FX (Beckman) instrument, and images were acquired as described above.

Fluorescence image acquisition and quantitative analysis

Images were analyzed using Image J. Translocation is assessed by calculating the nuclear/cytoplasmic (N:C) ratio of the mean fluorescence intensity for a population of cells (denoted as *n*) over several images for a given condition. In the IκBα-YFP[1]/YFP[2]-p65 assay, total mean fluorescence intensity ($\pm$SE) for all cells is assessed by adding weighted mean fluorescence intensities for the nucleus and cytoplasm for individual cells in the population for a given condition.

Luciferase reporter assays

HEK293T cells were seeded in 96-well poly-D-lysine-coated plates at 7,500 cells/well. The next day, cells were transfected with 100 ng of plasmid of $(NF\kappa B)_3$-luc, a plasmid containing three NFκB promoter elements upstream of a luciferase reporter[20] using LipofectAmine 2000 according to the manufacturer's instructions (Invitrogen). After 48 h, cells were pretreated for 90 min with varying doses of compounds, followed by 6 h of incubation in the absence or presence of 10 ng/ml human TNFα (Roche). The medium was replaced with PBS and Bright-Glo Luciferase Assay Reagent (Promega). Cell lysates were transferred to opaque flat-bottom assay plates, and luminescence was recorded on a Lumiskan Ascent (Thermo Labsystems) luminometer.

Results

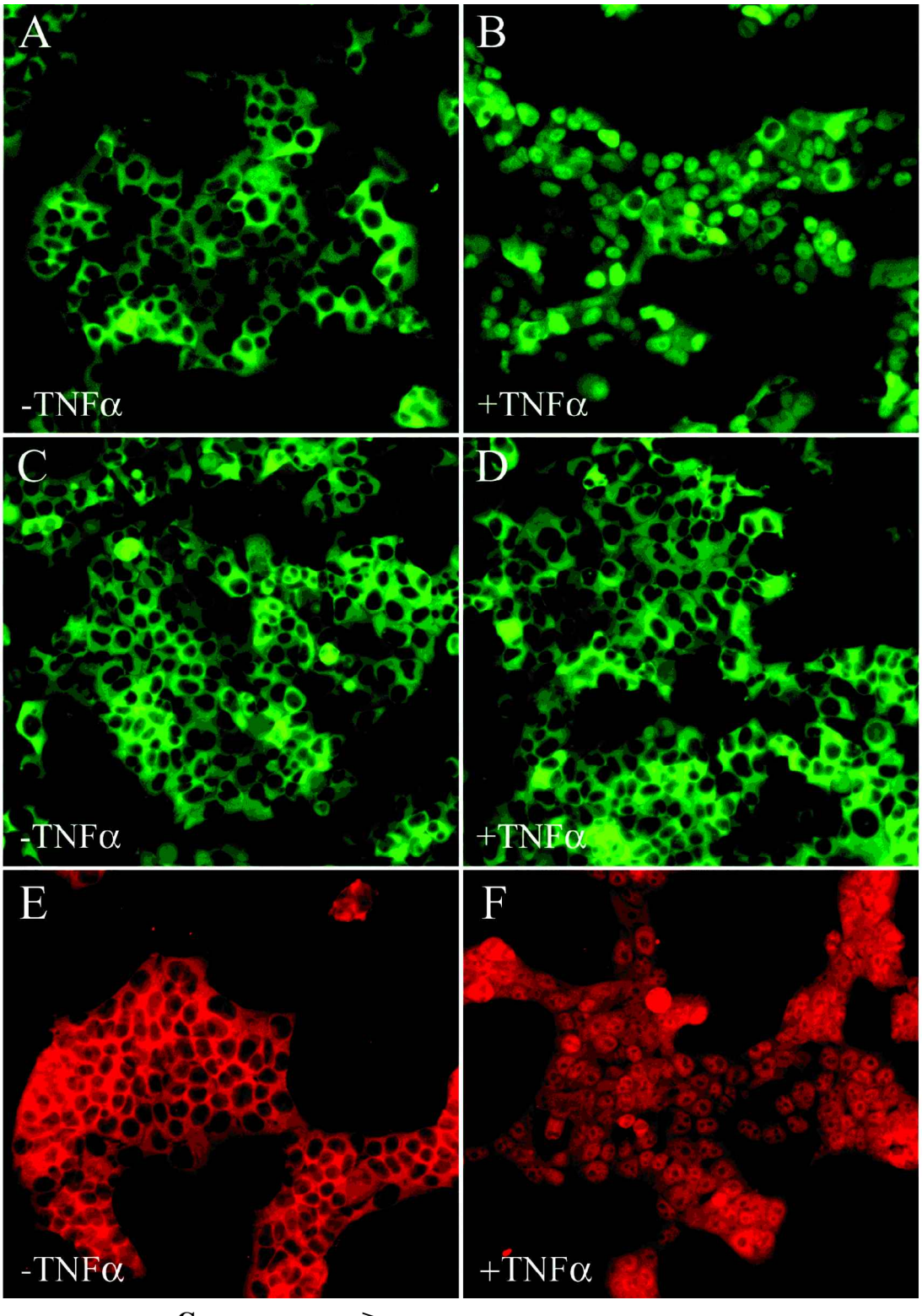
p50/p65 PCA development

Fragments of YFP were synthesized (Blue Heron, Inc.)[18] and fused via a 10-amino acid flexible linker to the N-terminal ends of p50 and p65. No YFP fluorescence was detected in HEK293T cells when YFP[1]-p50 and YFP[2]-p65 were expressed separately (data not shown). YFP[1]-p50 and YFP[2]-p65 were stably expressed in HEK293T cells. MEK-YFP[2] and ERK-YFP[1] transfected cells were generated as controls representing the activity of a different pathway. Cell clones stably expressing the fusion genes were identified by immunoblot analysis and fluorescence microscopy. A single cell line of each transfectant was selected for further characterization.

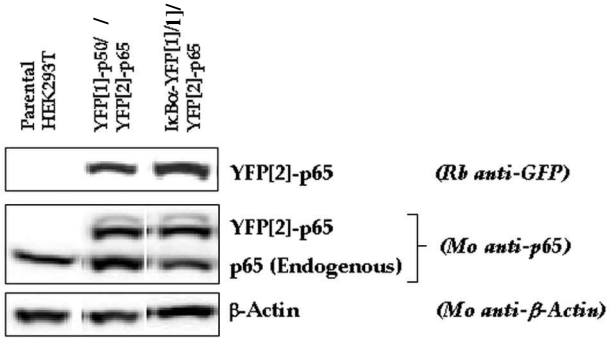
In unstimulated cells, p50/p65 complexes were located predominantly in the cytoplasm (Fig. 2A), consistent with the distribution of the endogenous proteins in unstimulated cells (Fig. 2E). TNFα treatment resulted in the translocation of p50/p65 complexes into the nucleus (Fig. 2B). In contrast, TNFα treatment did not affect the predominantly cytoplasmic localization of the MEK/ERK complexes (Fig. 2C and D). Analysis of endogenous p65 in the parental cells by immunofluorescence demonstrates a remarkably similar pattern of nuclear translocation following TNFα treatment (Fig. 2E and F). We noted that most, but not all, cells demonstrate nuclear translocation of NFκB complexes following TNFα treatment. Further examination indicates that cells actively exclude NFκB from the nucleus following mitosis (data not shown). This phenomenon is likely to explain the low-level heterogeneity in the translocation response in both PCA- and immunofluorescence-visualized NFκB. Using both gene-specific (anti-p65) and reporter-specific (anti-GFP) antibodies, we found that expression levels of the YFP[2]-p65 fusion protein were comparable to those of endogenous p65 in engineered cell lines and in the parental HEK293T cells, respectively (Fig. 2G). Our results indicate that, under conditions where PCA constructs are expressed at physiological levels, robust fluorescent signals are observed. We also found that these engineered cell lines demonstrate stable fluorescence over at least 20 passages (data not shown).

We further characterized the p50/p65 cell line by quantitative image analysis. The mean fluorescence of the nucleus and cytoplasm of individual cells was quantitated, and the N:C fluorescence ratio was calculated. Treatment

FIG. 2. TNFα induced nuclear translocation of NFκB p50/p65 complexes in live cells. Cells stably expressing YFP[1]-p50/YFP[2]-p65(**A** and **B**) or MEK1-YFP[2]/ERK1-YFP[1] (**C** and **D**) were incubated in the absence or presence of 50 ng/ml TNFα for 30 min as indicated. Existence and localization of p50/p65 heterodimers and MEK1/ERK1 complexes were detected by fluorescence imaging (40$\times$ objective). In unstimulated cells, p50/p65 complexes were localized in the cytoplasm (A). Following cytokine treatment, the heterodimers translocated into the nucleus (B). MEK1/ERK1 complexes remained in the cytoplasm in both unstimulated (C) and TNFα-stimulated cells (D). (**E** and **F**) Immunofluorescence of endogenous p65 in parental HEK293T cells. 293T cells incubated in the absence (E) or presence (F) of TNFα were fixed and stained with anti-p65 antibody followed by Cy3-labeled secondary antibody. Endogenous p65 observed in the cytoplasm of unstimulated cells was localized primarily in the nuclei following TNFα induction. (**G**) Stable expression of p65 PCA reporter in engineered cell lines. Proteins from parental cells, or cells stably expressing YFP[1]-p50/YFP[2]-p65 and IκBα-YFP[1]/YFP[2]-p65 were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, blotted, and probed with anti-GFP, anti-p65, and anti-actin antibodies as indicated. The anti-actin immunoblot serves as the loading control. The identity of the YFP[2]-p65 band was verified by using anti-GFP antibody, which recognized the YFP fragment-YFP[2] of the fusion protein (**top panel**).


A
-TNFα
B
+TNFα
C
-TNFα
D
+TNFα
E
-TNFα
F
+TNFα

G


Parental HEK293T
YFP[1]-p50/ YFP[2]-p65
IκBα-YFP[1]/1]/ YFP[2]-p65
YFP[2]-p65
(Rb anti-GFP)
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p65 (Endogenous)
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(Mo anti-β-Actin)

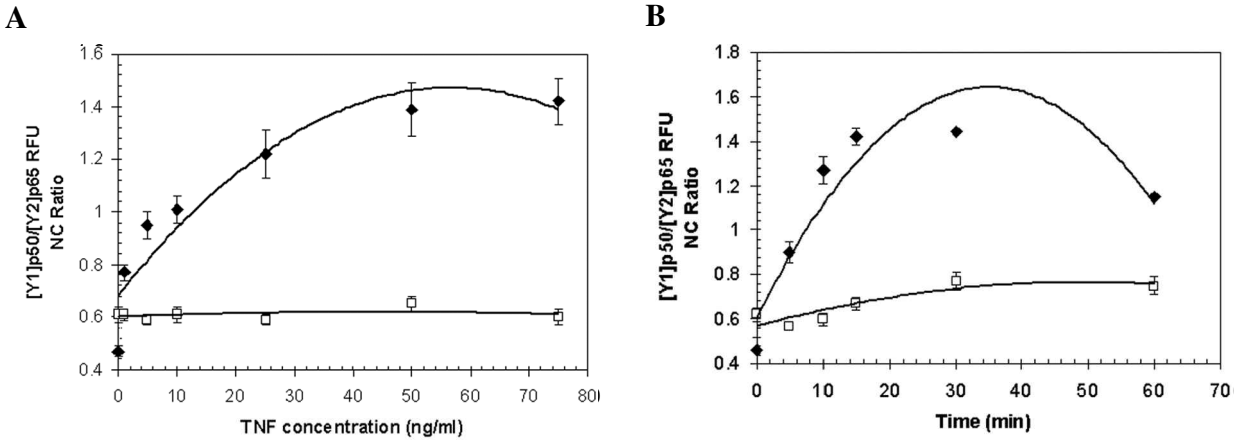

FIG. 3. Cytokine-induced nuclear translocation of p50/p65 complexes. (**A**) TNFα dose response. Stable p50/p65 PCA-engineered cells (filled diamonds) and control MEK1/ERK1 PCA-engineered cells (squares) were incubated with the indicated concentrations of TNFα for 30 min; $n = 100$ cells. (**B**) Time course of TNFα-induced p50/p65 nuclear translocation. p50/p65 cells (filled diamonds) or MEK/ERK cells (squares) were treated with 50 ng/ml TNFα for the indicated lengths of time. Mean fluorescence in the nucleus and cytoplasm was quantified by image analysis. Data are expressed as the ratio of nuclear to cytoplasmic fluorescence (N:C ratio; $n = 500$ cells). RFU, relative fluorescence units.

of the p50/p65 cell line with increasing doses of TNFα resulted in a threefold increase in the N:C ratio, from 0.47 to 1.42, with a half-maximal response at 10 ng/ml TNFα (Fig. 3A). Analysis of the time course of the TNFα response revealed that p50/p65 translocation into the nucleus occurred with a $t_{1/2}$ of 5 min (Fig. 3B). The maximal response was observed at 15 min, followed by a decrease at 60 min, consistent with feedback recovery of NFκB activation. TNFα had no effect on the control (MEK/ERK) PCA (Fig. 3).

The observed increase in N:C ratio of p65/p50 following induction by TNFα was identical to the magnitude of endogenous p65 nuclear translocation measured in parental cells (Fig. 4). Across the population of cells, the change in the N:C ratio of p50/p65 was highly significant ($p < 0.0001$). Analysis of four independent experiments demonstrated that the PCA response to TNFα was consistent (interassay CV = 5.9; data not shown). This assay functionally recapitulates with high fidelity the response of the endogenous transcription factors to pathway stimulation, and is a sensitive indicator of the TNFα signaling pathway.

IκB/p65 assay development

NFκB transcription factors are retained in the cytoplasm by IκB proteins. TNFα-induced degradation of IκB via ubiquitination and proteasomal degradation frees bound NFκB and leads to translocation of the transcription factor into the nucleus. Thus, disassembly of the IκB–NFκB complex is a key step in NFκB-mediated gene regulation. To visualize regulation of the NFκB pathway at this level, we engineered a stable cell line expressing an IκB/p65 PCA. Fluorescent imaging revealed that IκBα/p65 complexes were located predominantly in the cytoplasm (Fig. 5A). Treatment of the cells with TNFα resulted in a significant decrease in fluorescence (Fig. 5B), consistent with cytokine-induced proteolysis of IκBα and disassembly of IκBα/p65 PCA complexes. Western blot analysis of parental and IκBα/p65 PCA-engineered cells demonstrates that p65 is expressed at endogenous levels in this cell line (Fig. 2E). In addition, IκB-PCA fusion proteins are regulated by TNFα to an extent similar to the endogenous IκBα protein (Fig. 5C).

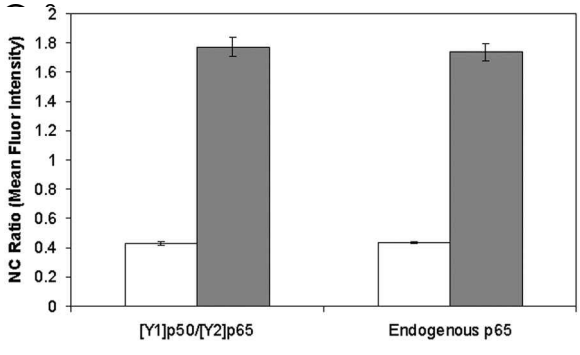

FIG. 4. Identical responses of p50/p65 PCA versus endogenous p65 to TNFα treatment. Stable p50/p65 cells grown in separate wells of a 96-well plate were incubated in the absence (-TNF; open bars) or presence (+TNF; filled bars) of cytokine for 30 min. Endogenous p65 in parental cells was detected by immunofluorescence in fixed, anti-p65 stained cells grown on coverslips in separate wells of a 12-well plate. The N:C ratios of YFP[1]-p50/YFP[2]-p65 complexes and endogenous p65 were calculated following quantitative fluorescence image analysis of 100 cells in each condition; means ± SE.

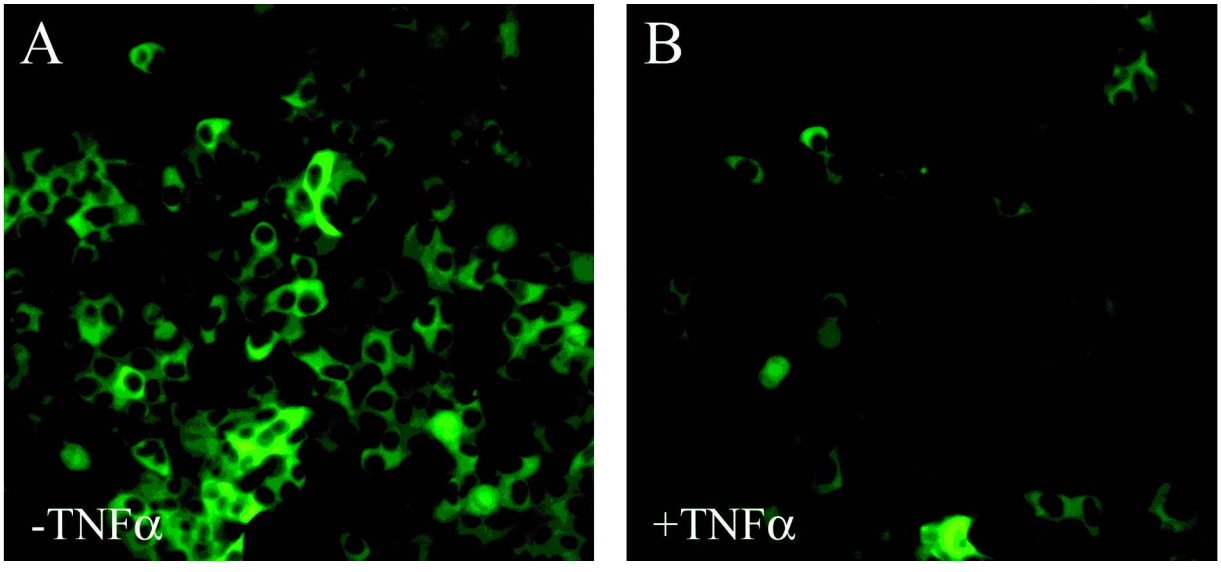

FIG. 5. Cells stably expressing IκBα-YFP[1]/YFP[2]-p65 were incubated without (**A**) or with (**B**) 50 ng/ml TNFα for 30 min. Complexes were localized in the cytoplasm of cells (A). (B) Fluorescence decreased in cells stimulated with TNFα. (**C**) PCA-IκBα is sensitive to proteasomal degradation. Parental and IκBα-YFP[1]/YFP[2]-p65 cells were incubated in the absence or presence of TNFα for 30 min. Lysates from these cells were analyzed for endogenous IκBα or IκBα-YFP[1] expression levels by immunoblotting with anti-IκBα or anti-GFP antibodies, respectively.

Quantitative image analysis showed a TNFα dose-dependent decrease in mean fluorescence intensities of IκBα/p65 complexes, but not of the control (MEK/ERK) complexes (Fig. 6A). This suggests that TNFα specifically induced the disassembly of IκBα/p65 complexes. The maximal response was observed at 10 ng/ml TNFα, where the mean cell fluorescence intensity of IκBα/p65 complexes was ~40% that of the unstimulated cells (Fig. 6A). Studies of the time course of the TNFα response showed a $t_{1/2}$ of 4 min, with a maximal response

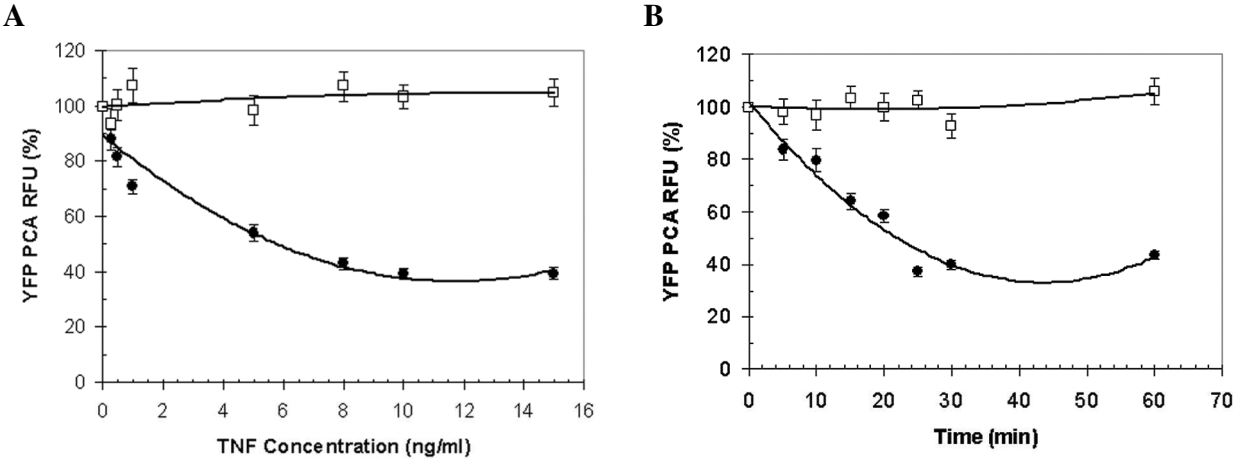

FIG. 6. Modulation of IκB/p65 complexes following pathway activation. (**A**) TNFα dose response. Stable IκBα-YFP[1]/YFP[2]-p65 cells (filled circles) and control MEK-YFP[2]/ERK1-YFP[1] cells (open squares) were incubated with increasing concentrations of TNFα as indicated (n = 500 cells). (**B**) Time course of TNFα-induced loss of IκBα/p65 complexes. IκBα-YFP[1]/YFP[2]-p65 (filled circles) and MEK-YFP[2]/ERK1-YFP[1] cells (open squares) were stimulated with 50 ng/ml TNFα for the indicated periods of time (n = 1000 cells). Mean cell fluorescence was measured by image analysis and expressed as a percentage of the value in unstimulated cells (all values shown are means ± SE). RFU, relative fluorescence units.

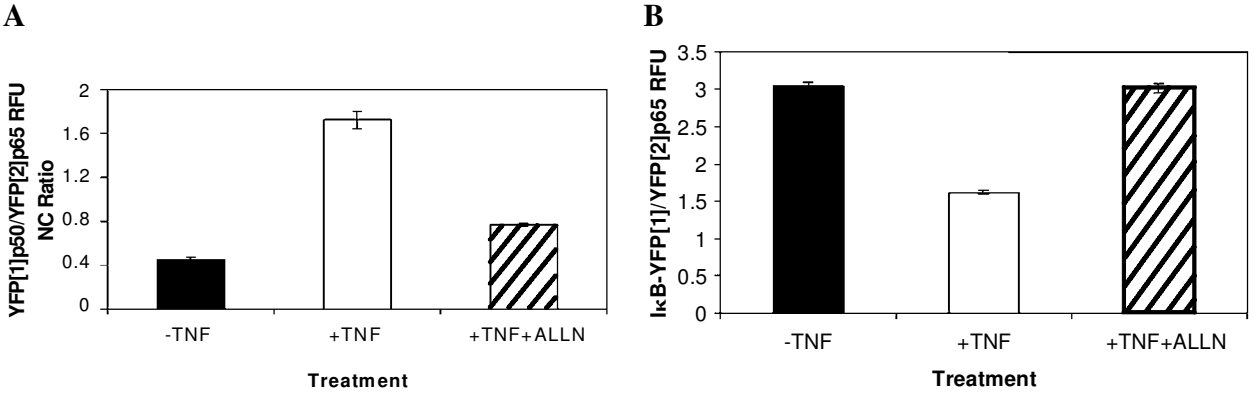

FIG. 7. PCA-engineered cell lines are suitable for high throughput drug discovery. p50/p65 PCA-engineered cells (**A**) and IκBα/p65 PCA-engineered cells (**B**) were treated with vehicle (−TNF), with TNFα (+TNF), or with TNFα following pretreatment with ALLN (+TNF+ALLN). Fluorescence intensity was measured and expressed as the N:C ratio (A) or relative fluorescence units (RFU) (B). All data are shown as means ± SE.

at 20 min. There was no effect of TNFα treatment on the fluorescence intensity of the control (MEK/ERK) PCA (Fig. 6). In sum, the kinetics of both the IκBα/p65 and p50/p65 responses are consistent with the responses of the endogenous proteins (Fig. 4, and data not shown), and indicate that the two signaling steps are tightly coupled. These results demonstrate that PCA is well suited to assessing dynamic regulation of signaling complexes in living cells.

Pharmacological modulation of PCAs

To determine if these assays are suitable for identification of novel inhibitors of TNFα/NFκB-dependent pathways, we first tested a known inhibitor of the pathway that acts upstream in these assays. The proteasome inhibitor ALLN was tested with both the p50/p65 and IkBα/p65 PCAs. ALLN treatment for 4 h blocked TNFα-induced increases in the N:C ratio of p50/p65 complexes by 76% (Fig. 7A). Likewise, ALLN inhibited the TNFα-induced reduction of IκBα/p65 complexes by 98% (Fig. 7B). These results demonstrate that NFκB and IκBα complexes visualized by PCA are regulated by TNFα signaling through ubiquitin/proteasome-mediated events, and further suggest that both will be sensitive assays for identification of novel therapeutics in an HTS setting. In addition, the different nature of the response in these two assays, and the fact that they probe sequential levels in the signaling cascade, provide complementary data sets for HTS and follow-up.

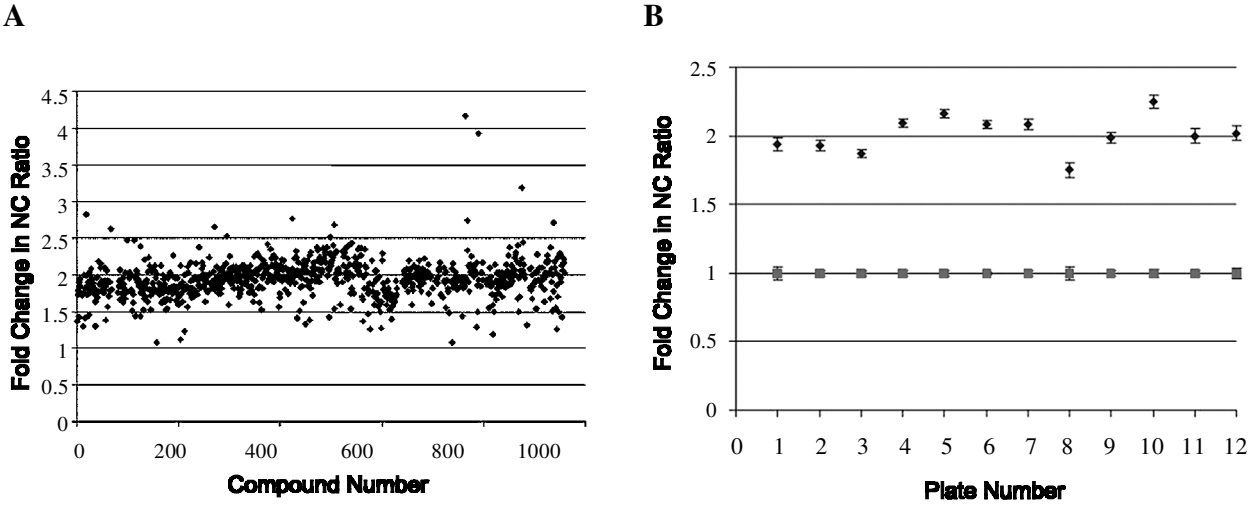

FIG. 8. Performance of p50/p65 PCA library screen. Data were obtained following treatment of live cells with 10 μM of each compound, followed by stimulation with TNFα (25 ng/ml final concentration). (**A**) Results of a 960-compound screen are shown, expressed as fold change in N:C fluorescence as determined by automated image analysis. (**B**) Plate performance across the screen; Z' values averaged 0.627. Diamonds, with TNFα; squares, without TNFα.

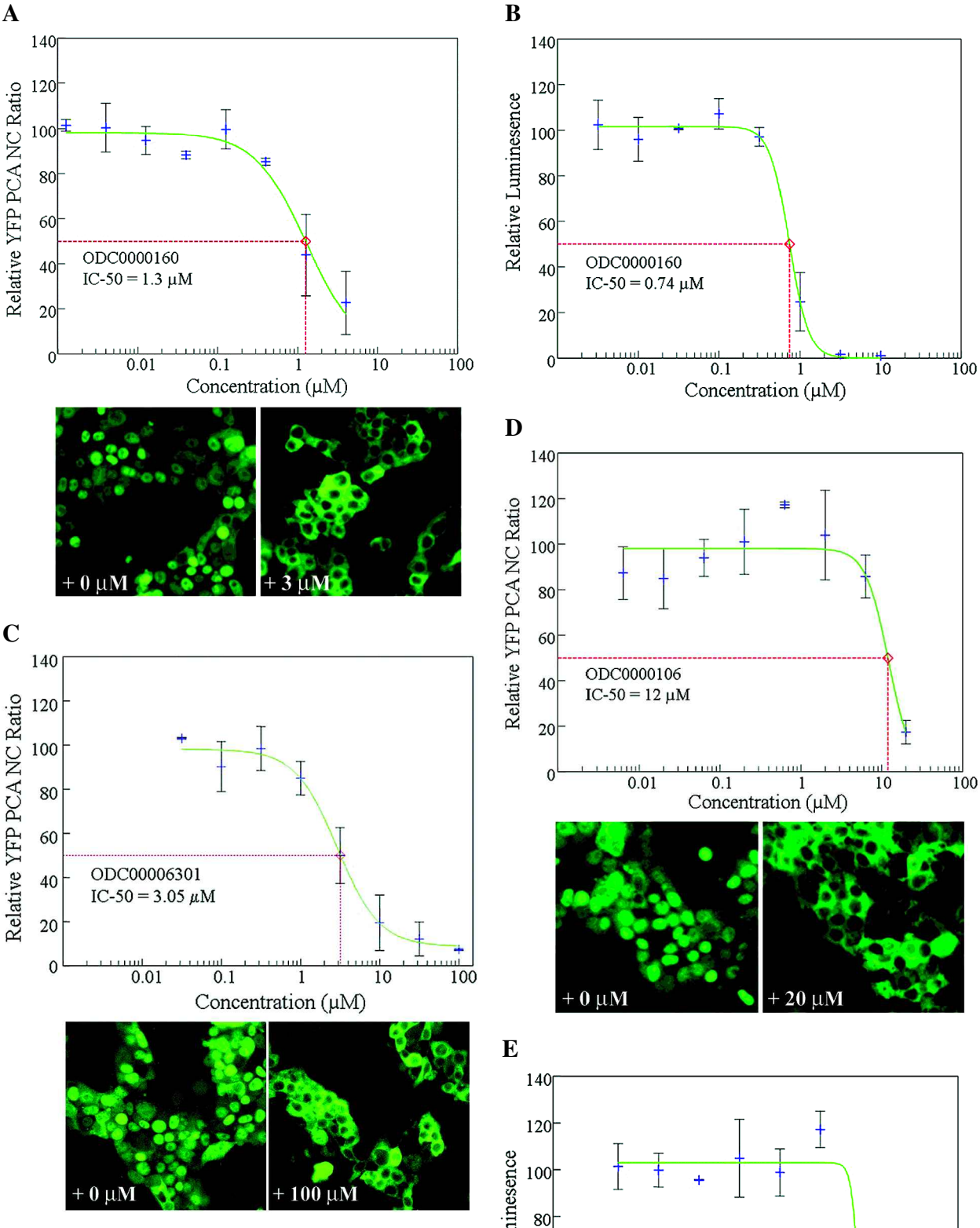

FIG. 9. Representative screening hits obtained with the live cell p50/p65 PCA. Eight-point dose-response hit confirmation was performed on selected compounds identified in the primary screen. IC_{50} values observed in PCAs compared favorably with compound activity in an NFκB transcription-based assay [compare **A** (PCA) with **B** (luciferase)]. Hits in the low micromolar range were identified and confirmed (**A** and **C**). Some dose-response curves (*e.g.*, ODC0000106, **D**) demonstrated steep Hill slopes, a characteristic of the compound that was confirmed in transcription-based assays (**E**). Example images quantitated in the dose-response curves are shown for A, C, and E.

Utilization of PCA for HTS

To determine if PCAs can be used in HTS, the Genesis Plus collection of compounds (Microsource Discovery Systems) was assayed in the cells engineered to express the p50/p65 PCA. Genesis Plus is a collection of 960 diverse compounds and includes compounds with known toxicity or fluorescent properties. Inclusion of compounds with such properties is important in HTS assay validation, as they might complicate analysis. The final concentration of compounds in wells was 10 μM, and all wells contained 0.5% dimethyl sulfoxide concentration. Cells were treated with compound (or vehicle) for 90 min, and then treated with 25 ng/ml TNFα for 30 min. Following fixation and staining of nuclei, fluorescence was analyzed on the automated fluorescent microscopy platform (Discovery 1; Molecular Devices Corp.).

The average N:C ratio was derived by automated image analysis as described above, and compound-treated wells were compared with unstimulated and TNFα-stimulated control wells. Results from this focused library screen are shown in Fig. 8A, and the plate-to-plate variability in TNFα response is shown in Fig. 8B. The Z factor, a commonly used metric for assay robustness, is not applicable for this subset of compounds due to the large number of known actives and fluorescent compounds. We utilized the Z' factor, which measures the same statistical parameter across control wells, to calculate assay quality (Fig. 8B).[21] The Z' values averaged 0.627, with a median value of 0.67 across the 12 assay plates. Fluorescent and toxic compounds were readily identified in the automated analysis of N:C ratio, demonstrating that compounds with these properties can be identified as false positives in screening campaigns (data not shown). Two compounds in this set known to affect the NFκB pathway, rotenone and 3-methylxanthine, were called as hits in the assay.[22,23]

In addition to the known inhibitors of this pathway in this compound set, we identified novel NFκB pathway inhibitors. For example, hit confirmation and eight-point dose-response analysis indicate that ODC0000160 inhibits the p50/65 PCA assay with an IC_{50} of 1.3 μM, relatively potent for a screening hit in a cell-based assay (Fig. 9A). A transcription-based readout of pathway activity gave a similar result (Fig. 9B). ODC0006301 was also identified as a cell-active inhibitor (Fig. 9C). Both of these compounds have been used in human clinical trials, but neither has been linked previously to the NFκB pathway. Clearly, their activity in this assay may have mechanistic significance, a concept supported by the fact that ODC0000160 can elicit apoptosis of human tumor cells (data not shown). Another compound identified as a hit in this assay, ODC0000106, is a known inhibitor of this pathway (BAY 11-7082). Dose-response analysis indicates that this compound is a weaker inhibitor in the PCA and luciferase assays, and yields a steeper Hill slope than other inhibitors (Fig. 9D and E). This compound is known to be an irreversible inhibitor of the IKKβ enzyme.[24] The simultaneous exclusion of toxic compounds, enabled by the analysis of cell number and nuclear morphology via Hoechst staining in the standard protocol, provides added confidence to hits obtained in these assays.

Discussion

Pharmaceutical company investment in new drug discovery and development has increased dramatically over the last 10 years, yet the rate of new drug approvals has not kept pace. Expensive preclinical and clinical failures are to blame for much of the inefficiency of the current process. Earlier application of pathway-based approaches to therapeutic identification and lead profiling can improve the success rate of drug discovery. PCA is an ideal strategy for rapidly and quantitatively identifying modulators of specific signaling processes in living cells.

We have constructed PCAs that assess the activity of the TNFα/NFκB pathway. These assays are robust, scalable, and quantitative. The response of the PCA assay signaling complexes described here closely mirrors the activity and subcellular localization of their endogenous protein counterparts (Figs. 2 and 4). Importantly, the expression of low levels of PCA constructs—even at or below endogenous protein expression levels—still yields sufficiently intense fluorescent signals for analysis and HTS. A previous analysis of p50/p65 subcellular localization by fluorescence PCA reported exclusively nuclear localization in Cos-1 cells.[25] This discrepancy with the expected regulation of NFκB and with our results described above (Figs. 2–4) is easily accounted for by differences in experimental conditions. We have observed marked effects of DNA concentration on subcellular localization in transiently transfected cells (data not shown). NFκB is actively retained in the cytoplasm of unstimulated cells by IκB. The high level of p50/p65 expression in transiently transfected cells perturbs the balance between the transcription factor and its modulator. Excess p50/p65 complexes not bound to IκB freely translocate to the nucleus of these cells. Not surprisingly, therefore, significant overexpression of p50/p65 described previously[25] resulted in their mislocalization to the nucleus. Utilization of bright fluorescent PCA reporters, and optimal cell line engineering, enabled analysis of cells expressing p50/p65 proteins at physiological levels with the proper subcellular localization (Figs. 2–4).

Engineering of stable PCA cell lines for p50/p65 and IκBα/p65, and assay optimization and formatting for HTS, were straightforward and have been accomplished for numerous other PCAs in our laboratories. For exam-

ple, several other signaling nodes in the NFκB pathway, as well as other pathways and target classes (*e.g.*, G protein-coupled receptors, kinases, nuclear hormone receptors), have yielded robust responses to known agonists and antagonists (data not shown). It is important to note that functionally different classes of targets can be probed using PCA. In the studies described herein, we constructed a "high content" assay measuring the dynamics of NFκB transcription factor in subcellular localization. The IκBα/p65 assay can also be analyzed by automated microscopy methods; alternatively, due to its ability to measure changes in total cell (and therefore per-well) fluorescence intensity, standard fluorescence plate readers can be used.

The most striking aspect of the PCA approach is the ability to detect dynamic changes in signaling complexes in live cells. Traditionally, these changes have been deduced from biochemical extracts, or visualized in fixed cells.[16] More recently, fusions of full-length fluorescent reporters to single proteins, including p65, have yielded important data.[26–28] In some cases, PCA may yield data similar to these traditional approaches. For example, we find (Figs. 2 and 4) that analysis of p65 translocation by immunofluorescence gives a result similar to PCA analysis of p50/p65 translocation. However, the outcome of signaling depends to a great extent on the composition of specific complexes (such as p50/p65 versus p50/p50 transcriptional dimers) formed by individual proteins, as well as the proteins they associate with during sequential steps of the signal transduction cascade.[29] Therefore, the ability to assess the regulation and translocation of particular protein complexes, as they normally occur in dimeric or multimeric context, should facilitate discovery of drugs with a high level of specificity. The use of PCA to visualize functionally unique protein complexes represents a significant advancement over traditional methods of labeling single proteins.[26] In addition, the absolute number of dynamic, single protein assays is certainly more limited than the number of assays assessing protein complex dynamics.

The high fidelity to endogenous signaling activity and robust performance of these assays led us to test their utility for compound library screening. We performed a proof-of-concept library screen on the p50/p65 PCA. Pharmacological action of some compounds known to act on this path closely mirrors results obtained in traditional assay formats, indicating the robustness of the PCA screen. On the other hand, some compounds were identified that were not active in other formats (data not shown), indicating the uniqueness of these assays. In addition, the discovery of well-known compounds that were previously not linked to this pathway suggests that these information-rich assays will identify novel modulators, even in heavily assayed chemical space, including libraries of marketed therapeutics (Fig. 9A and C, and data not shown). Advantages of this cell-based screening approach include the simple exclusion of cytotoxic or fluorescent compounds. The kinetics of these assays allow rapid assessment of signaling activity, decreasing the likelihood of compounds acting through secondary mechanisms being identified as hits. One of the major challenges of all cell-based assays is determination of compound mechanism of action. Constructing a set of PCA assays encompassing the signaling pathway allows rapid identification of the proteins in the pathway that are regulated by a compound of interest. Further, utilizing PCA assays from other pathways is a powerful tool for identifying unanticipated, off-pathway effects of hits and lead series.

By probing entire pathways (and their connections) in living cells, no assumptions need to be made as to the optimal target in a given pathway. The cell will "select" the most efficacious compounds in a library, and these will be either the most potent compounds or those compounds targeting the key signaling proteins (or both). Capturing a number of potential targets in a single screen represents an economy of scale, where more validated hits can be derived from fewer screens. Our results suggest that this approach represents a significant addition to the current arsenal of drug discovery technologies.

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