A Quantitative Single-Cell Assay for Protein Kinase B Reveals Important Insights into the Biochemical Behavior of an Intracellular Substrate Peptide†

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ABSTRACT: The introduction of peptides into living cells for the purpose of manipulating cellular biochemistry has become widespread throughout biology. However, little is known about the behavior of these short sequences of amino acids within cells, particularly those used as substrates or inhibitors for kinases and other enzymes. We utilized a quantitative, single-cell assay to demonstrate that an 11-amino acid peptide was efficiently phosphorylated by intracellular protein kinase B (PKB) in fibrosarcoma cell line HT1080 and in NIH-3T3 cells. The phosphorylated peptide was also readily dephosphorylated by intracellular phosphatases. Assays of the peptide's phosphorylation in single, living cells measured the balance of the activities of PKB and phosphatases in that cell. At a peptide concentration below the K_M of PKB and the phosphatases, the ratio of phosphorylated to nonphosphorylated peptide at the steady state was independent of the peptide concentration. A single-cell assay utilizing this peptide revealed the existence of two subpopulations of cells whose unique activities had hitherto been obscured by population averaging. Additional studies of cells stimulated by PDGF demonstrated that a quantitative analysis of PKB activation in response to a physiological stimulus was possible. These studies demonstrated that short peptides can remain specific within the complex intracellular milieu and function as sensitive reporters of the activation state of native kinases within live cells.

Peptides are being increasingly used in the study of cellular biochemistry, and recent years have seen a rapidly expanding repertoire of peptides possessing biological activity derived from native proteins or from combinatorial libraries (*1*−*3*). *In vitro* assays utilizing peptides as kinase substrates have been in use for more than a decade (*4*−*7*). In addition to their roles in kinase assays, numerous peptides have become available for use as inhibitors of kinases, in both the *in vitro* and most notably intracellular settings. For example, a number of cell-permeable pseudosubstrate peptides have been widely used to inhibit a variety of intracellular kinases (*2*, *8*−*10*). Small peptides based on protein interaction domains are proving to be effective inhibitors through the specific disruption of protein−protein interactions, including those of the mitogen-activated protein kinases (MAPKs)[1] and their upstream MAPK kinase (MEK) activators, as well as a variety of other kinases and signaling proteins (*11*−*15*). Most exciting is the therapeutic potential for intracellular, biologically active peptides (*10*, *15*, *16*). Thus, an improved understanding of peptide biochemistry within the living cell is needed as a growing number of techniques show the value of these molecules for the biological and pharmaceutical sciences.

There are a number of important caveats when peptides are used in live cells. While much is known about how peptides accumulate in and are eliminated from the organism, less is known about the intracellular behavior of these molecules (*16*). The low permeability of cell membranes to most peptides is one obstacle to their use and study, but new methods of delivery, particularly the use of protein transduction domains, are overcoming this problem (*17*). Cells contain a number of cytosolic endo- and exopeptidases which may degrade free peptides, thus limiting their intracellular lifetimes (*3*). Another critical issue is the specificity of a small peptide for a particular protein (*4*, *18*, *19*). Such peptides lack the tertiary structure and accessory domains of full-length proteins which may affect their localization and limit their interactions with intracellular targets (*3*). This issue is particularly important with the use of peptides as enzymatic substrates where intracellular localization and protein interaction domains are believed to be critical for conferring specificity (*12*, *13*).

Protein kinase B (PKB), also known as Akt, is one of many kinases for which peptide substrates and inhibitors have proven to have value for *in vitro* assays (*4*, *18*, *20*). This

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[1] Abbreviations: PKB, protein kinase B; LMS, laser micropipet system; CACE, cell activity by capillary electrophoresis; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PKC, protein kinase C; BSA, bovine serum albumin fraction V; PDGF, platelet-derived growth factor; α-NAP, α-naphthyl acid phosphate; ECB, extracellular buffer; CE, capillary electrophoresis; DMEM, Dulbecco's minimal essential medium; FCS, fetal calf serum; F-PKB, fluorescein-labeled substrate peptide for PKB; S6K1, p70 S6 kinase; MAPKAP-K1, MAP kinase-activated protein kinase-1; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; SGK, serum- and glucocorticoid-regulated protein kinase; FRET, fluorescence resonance energy transfer.

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serine/threonine kinase is of central importance in cellular metabolism and survival and has been found to be overexpressed in a number of human cancers (*21*). The mechanisms and regulation of PKB activation have been the subject of intense research over the past decade, and numerous reviews have been published that will provide the reader more detail than is possible here (*22*, *23*). Measurements of the intracellular activity of this kinase continue to be of intense interest to a wide variety of biologists, biomedical researchers, and pharmaceutical scientists. Peptides based on sequences derived from native substrates or from combinatorial screening have been shown to be readily phosphorylated by PKB *in vitro* (*4*, *18*, *20*). In the current study, quantitative, single-cell assays were undertaken to reveal the biochemical properties of a substrate peptide for PKB within the complex intracellular milieu. The value of these single-cell studies was demonstrated by revealing differences in PKB activity in individual cells that could not be observed in population-averaged measurements. In addition, the ability of the peptide to quantitatively report the activity of native PKB in cells stimulated by a physiological ligand has also been demonstrated. The technique and findings described in the current work will be broadly applicable to the study of intracellular kinases and phosphatases as well as to other applications in which peptides are being increasingly used as intracellular probes.

EXPERIMENTAL PROCEDURES

Reagents. All fluorescent reagents were obtained from Molecular Probes (Eugene, OR). Geneticin (G-418 sulfate) and all tissue culture materials were obtained from Gibco-BRL (Gaithersburg, MD). γ-Methacryloxypropyltrimethoxysilane, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, ammonium persulfate, bovine serum albumin fraction V (BSA), and wortmannin were obtained from Sigma. Acrylic acid (99% solution) was obtained from Aldrich. Fused-silica capillary tubing (30 μm inside diameter, 365 μm outside diameter) was from Polymicro Technologies Inc. Platelet-derived growth factor BB (PDGF) was purchased from Upstate Biotechnology (Lake Placid, NY). Rapamycin, α-naphthyl acid phosphate monosodium salt (α-NAP), PD098059, Gö 6983, and platelet-derived growth factor BB (PDGF) were purchased from Calbiochem (San Diego, CA). All other reagents and materials were purchased from Fisher Scientific (Pittsburgh, PA). A physiologic extracellular buffer (ECB) was composed of 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, and 1 mM $CaCl_2$, and adjusted to pH 7.4 with NaOH.

Fluorescent Peptide Synthesis. The PKB substrate peptide, GRPRAA**T**FAEG, was synthesized with an amidated C-terminus in both nonphosphorylated and phosphorylated forms by the Beckman Peptide and Nucleic Acid Facility at Stanford University (Stanford, CA). The peptide with fluorescein covalently bound to the N-terminus was prepared by incubating resin-bound peptide with 6-carboxyfluorescein, hydroxybenzoltriazole, and diisopropylcarbodiimde in dimethyl formamide at a molar ratio of 1:5:5:5 for 2 h on a rotating mixer at room temperature (*24*). The peptide was washed with dimethylformamide and ethyl acetate and then cleaved from the resin by incubation for 2 h with a degassed trifluoroacetic acid/free radical scavenger mixture [88% (v/v) trifluoroacetic acid, 5% (v/v) anisol, 5% (v/v) water, 2% (v/v) triisopropylsilane]. The peptide was separated from the resin with a sintered glass filter, ether precipitated, dried in air, and dissolved in 55% dimethylformamide in H_2O. The peptide was purified by reverse-phase HPLC, and molecular weight and peptide purity were assessed by MALDI-MS (PE Biosystems Voyager System 4124) and capillary electrophoresis (CE), respectively. The concentration of the fluorescein-labeled peptide was determined by amino acid analysis at the Molecular Structure Facility at the University of California (Davis, CA) by spiking the sample with a standard with a known concentration.

Cell Culture and Handling. The HT1080-6TG cell line contains one mutant and one wild-type *N-ras* allele, resulting in constitutively active signaling proteins downstream of Ras, including PKB (*25*−*27*). A second cell line derived from HT1080 (HT1080/PTEN) has been molecularly engineered to overexpress the PTEN phosphatase, resulting in greatly decreased PKB activity (*26*). HT1080/PTEN cells were maintained in medium containing the selective antibiotic Geneticin (800 μg/mL). HT1080, HT1080/PTEN, and NIH-3T3 cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37 °C and 5% CO_2. Cells used in experiments were grown in a 500 μL cell chamber made by using Sylgard 184 (Dow Corning, Midland, MI) to attach a silicon O ring (24 mm outer diameter) to a 25 mm, round, no. 1 glass coverslip. Cells were plated at a density of 5−10 cells per high-power field. Prior to being used, the cells were allowed to grow in supplemented media for 12−24 h after being plated in the cell chamber. Cells were then serum starved with DMEM containing 0.25% FCS for 18−24 h prior to experiments. NIH-3T3 cells used for PDGF stimulation were serum starved with DMEM lacking FCS overnight prior to experiments. All experiments were conducted at 37 °C. Immediately before the start of experiments, the medium in the cell chamber was exchanged with ECB. Throughout the course of the experiments, the buffer was constantly passed (1−4 mL/min at 37 °C) through the cell chamber. Many of the experiments were designed to study differences in peptide phosphorylation in cells with active or weakened and inactive PKB. Such comparisons were performed under both conditions on the same day rather than using historical data from prior experiments. Due to cellular heterogeneity, this design resulted in small variations in the average phosphorylation of the fluoresceinated substrate peptide for PKB (F-PKB) reported in different experiments under apparently identical conditions (e.g., serum-starved HT1080 cells loaded with F-PKB).

Western Blot Analysis. Subconfluent HT1080 cells were serum starved for 24 h. Levels of PKB and p70 S6 kinase (S6K1) phosphorylation were tested in cells treated for the indicated time with 100 nM wortmannin and 200 nM rapamycin, respectively. Cells were lysed in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 25 mM NaF, and 1 mM PMSF for 20 min on ice. Lysates were centrifuged at 13000*g* for 10 min at 4 °C, and the protein concentration of the supernatant was measured with a BCA protein assay kit (Pierce). Samples containing 80 μg of total protein were electrophoretically separated via 8% SDS−PAGE and transferred onto an

Immobilon-P transfer membrane (Millipore). The membrane was blocked in ST buffer [20 mM Tris (pH 7.5), 150 mM NaCl, and 0.1% Tween 20] containing 5% nonfat milk for 1 h. Rabbit anti-phospho Akt (Cell Signaling) and rabbit anti-phospho-p70 S6 kinase (Thr 412) (Upstate) antibodies were used according to the manufacturers' recommendations (overnight incubation at 4 °C). The membrane was washed three times with ST buffer for 5 min, followed by incubation with horseradish peroxidase-conjugated anti-rabbit Ab (1:10000, Santa Cruz Biotechnology) for 1 h at room temperature. After the membrane had been washed three times with ST buffer for 5 min, the immune complexes were detected with SuperSignal Chemiluminiscent Substrate (Pierce).

Use of Inhibitors. Wortmannin, a potent cell-permeable phosphoinositide 3-kinase (PI3K) inhibitor, was stored in DMSO (10 mM) at −20 °C in small aliquots. Aliquots were defrosted and used immediately. A working solution was prepared by dilution to 100 nM in DMEM, added to the cells, and incubated for 10 min at 37 °C (*28*, *29*). The broad spectrum phosphatase inhibitor α-NAP was microinjected into HT1080 cells along with the substrate peptide at a concentration of α-NAP in the injection pipet of 50 mM (*30*). After incubation for 10 min, individual cells were analyzed (see below). Rapamycin, an inhibitor of mTOR that blocks the activation of S6K1, was dissolved at 200 nM in DMEM, added to the cells, and incubated for 2 h at 37 °C (*31*, *32*). Cells were then loaded with the peptide followed by analysis. In some experiments, the activation of MAP kinase-activated protein kinase-1 (MAPKAP-K1) was blocked using the MEK inhibitor PD098059 (100 μM) for 90 min at 37 °C (*33*). Gö 6983 was used to inhibit protein kinase C (PKC), particularly isoforms α, β, γ, δ, and ζ (*34*, *35*). Gö 6983 (10 mM in DMSO) was diluted to a final concentration of 100 μM in DMEM, added to the cells, and incubated for 20 min at 37 °C (*35*). Following incubation with each inhibitor, cells were loaded with the substrate peptide and analyzed.

Stimulation of NIH-3T3 Cells with PDGF. A 10 μg/mL stock solution of PDGF-BB, human recombinant homodimer, in phosphate-buffered saline containing 0.1% BSA was prepared per the manufacturer's directions, aliquoted, and stored at −20 °C until it was used. A working solution was prepared on the day of the experiment by diluting the stock solution in ECB to the desired final concentration. During an experiment, a single cell was loaded with F-PKB by microinjection followed by flushing of the cell chamber with the PDGF-containing solution and incubation at 37 °C for 20 min. After the incubation period, the single cell was immediately analyzed.

Introduction of Peptide by Microinjection or Pinocytic Loading. Peptides were introduced into cells with either a commercial microinjection system (Transjector 5246, Eppendorf, Westbury, NY) or a pinocytic cell loading procedure (osmotic delivery) (*36*). Microinjection pipets were pulled to produce tips with an outside diameter of 0.5 μm (model 700C pipette puller, David Kopf Instruments, Kent, U.K.). Pinocytic loading was accomplished with the "Influx Pinocytic Cell-Loading Reagent" (Molecular Probes) and was performed per the manufacturer's protocol with minor modification as follows. Step 1 is hypertonic induction of pinosomes. Cells were loaded with peptide in hypertonic loading medium [polyethylene glycol and sucrose dissolved in buffer (ECB, pH 7.4) as per manufacturer] for 10 min at 37 °C. Step 2 is hypotonic disruption of pinosomes. Cells were placed in hypotonic medium (1:1 DMEM/water mixture) for 2 min at 37 °C. Step 3 is recovery of cells. Cells were recovered in ECB with 1% BSA for 10 min. The function of the BSA in the recovery media was to displace the nonspecifically adsorbed peptide in the dish. Prior to analysis, the cells were washed with ECB alone.

Cell Lysates for CE Analysis. A confluent flask of HT1080 cells was trypsinized and then resuspended in ECB at a density of 1×10^6 cells/mL. The cells were lysed by rapid freezing and thawing and then centrifuged at 1600*g* for 10 min, and the supernatant was collected.

Laser Micropipet System. The laser micropipet system (LMS) is composed of an inverted microscope coupled to a pulsed Nd:YAG laser and a capillary electrophoresis system (*37*). This system is now called CACE (cell activity by capillary electrophoresis) for consistency with the naming of other analytical technologies using chemical separations. The LMS/CACE was utilized as previously described (*38*, *39*).

Poly(acrylate)-Coated Capillaries. The inner surfaces of capillaries were coated with poly(acrylate) using a modified method of Hjerten and Kubo (*40*, *41*).

Capillary Electrophoresis. CE was performed as described previously with the following exceptions (*37*). All analyses were performed using poly(acrylate)-coated capillaries (75 cm long with an optical window 48 cm from the inlet). The outlet was held at a negative potential of 21 kV, and the inlet reservoir was held at ground potential. Under these conditions, the current through the capillary was typically 36 μA. Solutions of standards were loaded into the capillary by gravitational fluid flow. The volume loaded was calculated from Poiseulle's equation and from contributions by spontaneous fluid displacement and diffusion (*42*−*44*). To estimate the intracellular concentration of the peptide, the number of moles contained in the cell was determined by comparison of the peak area from the electrophoretic trace of the cell versus that of the standard performed on the same day. A cell volume of 1 pL and 100% peptide recovery were assumed in calculating the intracellular peptide concentration (*37*, *38*). Analytes were detected by laser-induced fluorescence (LIF) as described previously (*37*). The buffer (ECB) surrounding the cell to be sampled was identical to that contained in the capillary during sample loading and electrophoresis.

RESULTS

For the assay of intracellular PKB, a fluorescein-labeled peptide (fluorescein-GRPRAA**T**FAEG, herein abbreviated F-PKB) based on the substrate RPRAATF was synthesized and introduced into cells (*4*). To determine the fate of the peptide in cells and its performance as a substrate, cells loaded with F-PKB were analyzed with LMS/CACE. This biochemical assay involves introducing a fluorescently labeled, peptide-based substrate (reporter) for a kinase or other enzyme (e.g., F-PKB) into cells by microinjection or pinocytic uptake. The catalytically active, native kinase(s) phosphorylates the reporter, and at the desired time, a cell containing the reporter is rapidly lysed by a shock wave generated with a pulsed Nd:YAG laser. The cell's contents are loaded into a capillary in such a manner that biochemical

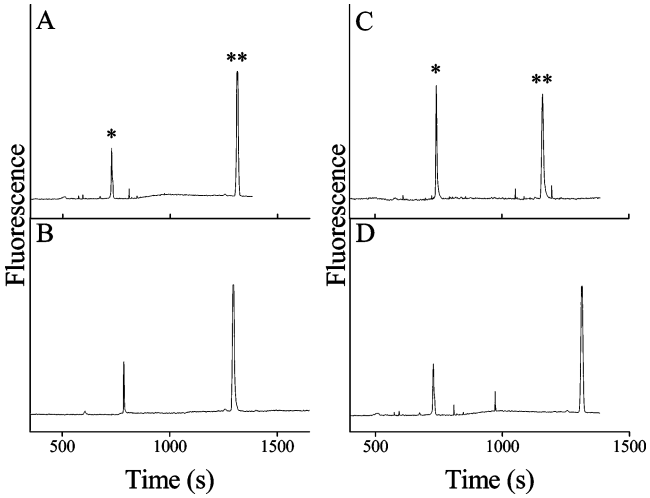

FIGURE 1: Electrophoretic analysis of F-PKB from single HT1080 cells. (A) Electrophoretic trace of a mixture of F-PKB (6.1×10^{-20} mol) and PF-PKB (2.1×10^{-19} mol) in buffer solution. (B) HT1080 cells were loaded with F-PKB by microinjection. After a 10 min recovery, a single cell was sampled and analyzed via LMS/CACE. Shown is a typical electrophoretic trace from a single-cell analysis using the same capillary as described for panel A. (C) Electrophoretic trace of a mixture of F-PKB (2.1×10^{-19} mol) and PF-PKB (1.2×10^{-19} mol) in buffer solution using a capillary different from that used for panels A and B. (D) Electrophoresis trace in the same capillary as in panel C obtained from a single HT1080 cell loaded with F-PKB. Asterisks (one for F-PKB and two for PF-PKB) denote the peaks corresponding to the respective standard.

reactions are terminated on millisecond time scales (*37*−*39*). Until the moment of lysis, the cell is undisturbed and resides in a physiologic buffer solution (ECB). Phosphorylated and nonphosphorylated forms of the reporter are separated by CE and detected by laser-induced fluorescence detection. The number of moles of substrate and product obtained from the cell can be determined from the electropherogram by comparison of their peak areas to standards. The ratio of the product to substrate provides a quantitative "snapshot" of the activity of the measured kinase at the time of the analysis.

F-PKB was introduced into serum-starved cells by microinjection or by pinocytic loading followed by an incubation period during which the peptide was exposed to any activated kinases within the cell. The human fibrosarcoma cell line HT1080 utilized in these experiments possesses constitutively active Ras, and constitutive production of PDGF, leading to persistent activation of PKB (*25*, *26*). HT1080 cells were loaded with F-PKB, sampled, and analyzed with LMS/CACE to determine if the peptide was modified in the cell. Figure 1 shows electrophoretic traces from single cells in these experiments. As seen in panels A and B of Figure 1, electrophoretic traces from HT1080 cells showed two peaks, a small initial peak and a predominant second peak. The migration time of the initial peak corresponded to that of an F-PKB standard. In many, but not all, electropherograms ($n > 20$), the migration time of the second peak was the same as that of the phosphorylated F-PKB (PF-PKB) standard.

In those analyses using F-PKB where migration time differences were present on electrophoretic traces of cells compared with those of standards (Figure 1C,D), the magnitude of the difference in the migration times was

Table 1: Variability in Electrophoretic Migration Times of F-PKB and PF-PKB

	migration time ± standard deviation (s)	
	F-PKB	PF-PKB
capillary 1		
standards ($n = 3$)	740 ± 9	1326 ± 12
cells ($n = 8$)	736 ± 7	1379 ± 13
capillary 2		
standards ($n = 3$)	745 ± 16	1170 ± 26
cells ($n = 6$)	749 ± 18	1381 ± 40
capillary 3		
prelysate ($n = 2$)	882, 882[a]	1575, 1559[a]
postlysate ($n = 6$)	925 ± 46	1771 ± 78

[a] Raw data shown because of the limited number of runs that were performed.

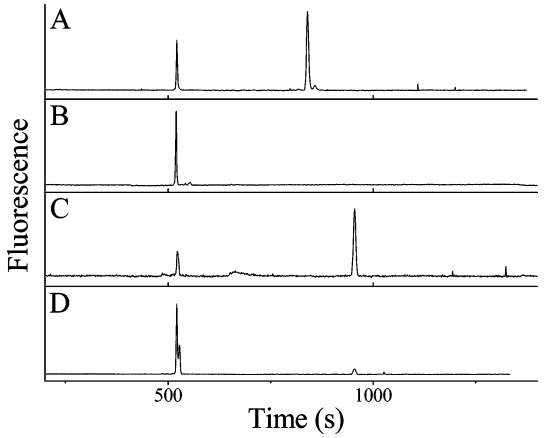

FIGURE 2: Measurement of PKB activity in NIH-3T3 cells. (A) Standard mixture of F-PKB and PF-PKB in buffer solution. (B) Electrophoresis trace obtained from a single NIH-3T3 cell. Microinjection was used to introduce F-PKB into the cell followed by a 10 min recovery prior to LMS/CACE analysis. (C) Electrophoresis trace from the analysis of an NIH-3T3 cell loaded with F-PKB as described for panel B followed by incubation with PDGF (10 ng/mL, 20 min). (D) Electrophoresis trace from the analysis of an NIH-3T3 cell incubated with wortmannin followed by loading with F-PKB and stimulation with PDGF as described for panel C.

capillary-dependent, and was more prevalent with the peak corresponding to PF-PKB (Table 1). In some capillaries, migration times for the peptides loaded into the capillary in buffer (standards) and the peaks obtained from cells were closely correlated (capillary 1 in Table 1 and panels A and B of Figure 1). In other capillaries, the differences in migration times were more marked (capillary 2 in Table 1 and panels C and D of Figure 1). When migration time differences were present, the second peak obtained from a cell invariably exhibited a longer migration time than the PF-PKB standard, but was never more than 120% of the standard's migration time. The run-to-run migration time of the second peak among different cells in the same capillary was highly reproducible with a percent standard deviation of only 2% (Table 1). This value was identical to the run-to-run variation among standards. The prolongation of the migration time of the second peak occurred irrespective of cell type (Figure 2). Since the sampling method lyses the cell and loads its entire contents into the capillary, it seemed plausible that the presence of cellular constituents could be influencing the migration time. To test this hypothesis, the supernatant from a lysate of HT1080 cells in ECB was

flushed through the capillary followed by a brief wash with run buffer. Standard runs before and after exposure of the capillary to the cell lysate revealed that the pattern of variability in migration time was similar to that seen in cell analyses (capillary 3 in Table 1). These data suggest that one or more constituents of the cell might have interacted with the capillary walls to alter the migration time of PF-PKB.

To determine if the second peak was the result of breakdown of the peptide within the cell, HT1080 cells were loaded by pinocytic uptake with a peptide in which the threonine residue of F-PKB was substituted with an alanine (GRPRAA-A-FAEG). LMS/CACE analyses of such cells showed a single peak with a migration time identical to that of the alanine-substituted peptide in buffer with no second peak (data not shown). This result suggested that peptide breakdown was unlikely to be occurring and was consistent with phosphorylation as the source for the second peak in F-PKB-loaded cells.

To confirm the identity of the second peak as that of phosphorylated F-PKB, NIH-3T3 cells loaded with F-PKB were analyzed. The NIH-3T3 cells used in these studies have been shown to display minimal phosphorylated PKB under serum-starved conditions using Western immunoblots with phospho-specific antibodies to S^{473} and T^{308} (*45*). Analysis of electrophoretic traces obtained from serum-starved NIH-3T3 cells loaded with F-PKB showed that in four of nine cells a single peak was present which comigrated with the F-PKB standard (Figure 2A,B). In the remainder of the cells, a second peak was present with a migration time similar to that of the second peak seen in HT-1080 cells, but much smaller in magnitude. On average, 94 ± 7% ($n = 9$) of the F-PKB loaded into serum-starved NIH-3T3 cells was present in the peak comigrating with the F-PKB standard (see Discussion). Analyses of NIH-3T3 cells incubated with PDGF (10 ng/mL) revealed an increase in the magnitude of the second peak with on average 89 ± 7% ($n = 4$) of the total F-PKB now present in this peak (Figure 2C). Analysis of NIH-3T3 cells preincubated with wortmannin followed by exposure to PDGF (10 ng/mL) revealed on average 93 ± 6% ($n = 4$) of the F-PKB comigrating with the F-PKB standard (Figure 2D). These data strongly support the identity of the second peak as phosphorylated F-PKB. In addition, this phosphorylation could be assessed after physiological stimulation of the cell.

To further verify that the second peak was the phosphorylated peptide, experiments were performed in HT1080 cells loaded with the phosphorylated peptide PF-PKB. After PF-PKB had been loaded into cells by microinjection, individual cells were analyzed via LMS/CACE within 2−4 min (Figure 3). An initial peak with an average migration time of 683 ± 10 s and a later peak with a migration time of 1158 ± 20 s ($n = 8$) were present. The migration times of F-PKB and PF-PKB standards in the same capillary were 689 ± 37 and 971 ± 23 s, respectively ($n = 3$). A similar pattern was seen in other cell types that were examined (data shown for only HT1080). Cells loaded with the phosphorylated form of the peptide exhibited a second peak with a prolonged migration time compared to that of the phosphorylated standard, confirming the identity of the second peak as PF-PKB. In addition, the appearance of the nonphosphorylated peptide on the electrophoretic trace suggested the presence of phosphatases acting on the peptide (see below). Analysis of HT1080 cells loaded with PF-PKB in which PI3K had been inhibited by exposure to wortmannin revealed nearly complete conversion of the PF-PKB to F-PKB [99.8 ± 0.7% as F-PKB ($n = 5$)] within 4 min of loading (Figure 3C).

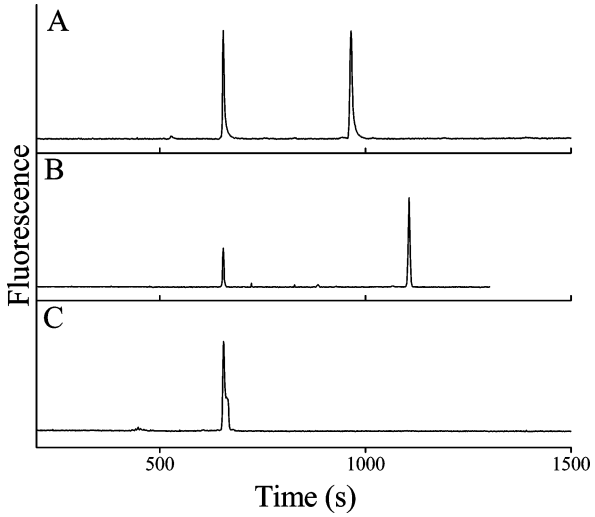

FIGURE 3: Analysis of an HT1080 cell loaded with the PF-PKB peptide. (A) Electrophoresis trace of a standard mixture of F-PKB and PF-PKB in buffer solution. (B) Electrophoresis trace obtained from a single HT1080 cell loaded with PF-PKB by microinjection followed by a 4 min recovery period. (C) Electrophoresis trace obtained from a single HT1080 cell preincubated with wortmannin, and then loaded with PF-PKB by microinjection followed by a 4 min recovery period.

To determine whether the phosphorylation of the peptide seen in HT1080 cells was the result of intracellular PKB activity, F-PKB was studied in single HT1080 cells under a variety of experimental conditions. On average, 59 ± 19% ($n = 17$) of the F-PKB loaded into HT1080 cells was phosphorylated within 10 min at 37 °C (Figure 4A). Overnight exposure of HT1080 cells to 10% serum did not increase the amount of PF-PKB in these cells [60 ± 18% ($n = 12$)], suggesting that PKB was fully active irrespective of serum starvation or growth factor (serum) stimulation. In HT1080 cells, all signaling pathways downstream of Ras are constitutively active (*25*, *26*). Thus, it was possible that another kinase could have phosphorylated F-PKB in these cells. Incubation of the cells with wortmannin reduced the percentage of PF-PKB to 27 ± 18% ($n = 4$) (Figure 4B). The HT1080/PTEN cell line was used to further test the specificity of phosphorylation of the intracellular peptide. HT1080/PTEN cells overexpress the dual-specificity phosphatase PTEN which catalyzes the dephosphorylation of phosphatidylinositol 3,4,5-trisphosphate (PIP_3). In previous work, we have shown that in these cells there is a dramatic reduction in the level of phosphorylation of PKB on Western immunoblot assays, while the multitude of kinases in other pathways remains constitutively active at levels equivalent to those in the parental HT1080 line (*26*, *46*). In the HT1080/PTEN cells on average, only 6 ± 10% ($n = 14$) of the F-PKB was phosphorylated (Figure 4C).

Two insulin-stimulated kinases, S6K1 and MAPKAP-K1 which have substrate motifs similar to those for PKB, have been shown to be capable of *in vitro* phosphorylation of peptides similar to F-PKB, such as the PKB substrate

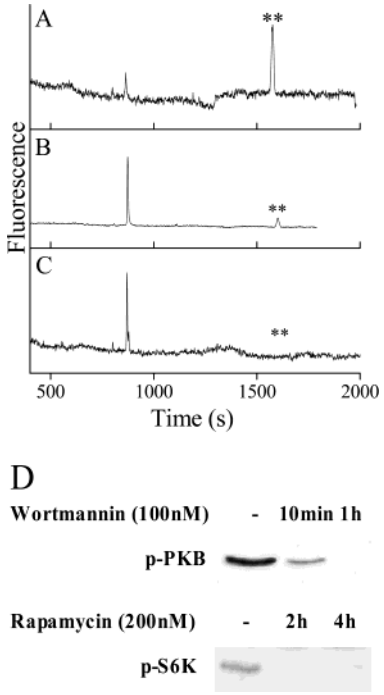

FIGURE 4: Analysis of kinase activity in HT1080 and HT1080/PTEN cells. Representative electrophoretic traces from the LMS/CACE analysis of cells loaded with F-PKB. The migration times were normalized on the basis of the migration time of the initial (nonphosphorylated) peak since the experiments were performed with different coated capillaries on different days. Two asterisks denote the migration time of PF-PKB from cells on the day of the experiment. (A) Electrophoretic trace of a single HT1080 cell loaded with F-PKB by pinocytosis and allowed to recover for 30 min. (B) LMS/CACE analysis of an HT1080 cell loaded as described for panel A and treated with wortmannin (100 nM). (C) Analysis of an HT1080/PTEN cell loaded with F-PKB by microinjection and allowed to recover for 10 min. (D) Western immunoblots of serum-starved HT1080 cells showing the time course of inhibition of the phosphorylation of PKB and S6K1 by wortmannin and rapamycin, respectively.

"Crosstide" (GRPRTSSFAEG), but do not phosphorylate the PKB substrate RPRAATF which is similar to, but not identical with, F-PKB (*4*). These kinases, as well as PKC, lie downstream of PI3K such that the reduced level of phosphorylation of F-PKB seen with wortmannin did not rule out their participation in phosphorylating a portion of the peptide. To determine whether these kinases were involved in the phosphorylation of F-PKB, serum-starved HT1080 cells were incubated with a series of pharmacologic inhibitors for these kinases. The mTOR inhibitor rapamycin blocks activation of S6K1, but not PKB (*31*, *32*). Western assays of rapamycin-treated HT1080 cells revealed a significant reduction in the level of phosphorylated S6K1 (Thr412) (Figure 4D). Under these conditions, analysis of cells via LMS/CACE showed that the percentage of phosphorylation of F-PKB was not reduced by rapamycin (Table 2); therefore, S6K1 did not appear to participate in the phosphorylation of F-PKB under these conditions. The MEK inhibitor PD098059 blocks activation of MAPKAP-K1 without inhibiting PI3K and PKB (*33*). We have previously shown in HT1080 cells that PD098059 inhibits the pathway leading to MAPKAP-K1 activation (*47*). Serum-starved HT1080 cells were incubated with PD098059, and then analyzed via LMS/CACE. PD098059 had no effect on the

Table 2: Effect of Inhibitors on F-PKB Phosphorylation

cell line	% F-PKB phosphorylated
HT1080	67 ± 18 ($n = 4$)
HT1080/PTEN	6 ± 10 ($n = 14$)
inhibitor in HT1080	**% F-PKB phosphorylated**
wortmannin	27 ± 18 ($n = 4$)
rapamycin	71 ± 11 ($n = 5$)
PD098059	65 ± 17 ($n = 4$)
Gö 6983	66 ± 18 ($n = 5$)

percentage of phosphorylation of F-PKB in these experiments (Table 2); therefore, MAPKAP-K1 did not appear to be participating in the phosphorylation of F-PKB under these conditions. Since some isoforms of PKC have been reported to have substrate specificity similar to that of PKB, experiments were also performed to confirm that PKC was not involved in F-PKB phosphorylation (*19*, *48*). Serum-starved HT1080 cells were incubated with the PKC inhibitor Gö 6983, and were then analyzed via LMS/CACE. As shown in Table 2, Gö 6983 had no effect on F-PKB phosphorylation, suggesting that under these conditions PKC was not involved in phosphorylation of F-PKB.

We next determined if the percentage of PF-PKB was related to the amount of peptide present in the cells. Although the final concentration of exogenous molecules loaded into cells cannot be finely controlled, the number of moles of peptide loaded in the cell can be determined from the LMS/CACE analysis by comparison of the peptide peak areas to those of standards (*37*, *38*). Serum-starved HT1080 cells loaded with a range of amounts of F-PKB from 8×10^{-21} to 6×10^{-19} mol were analyzed after incubation for 4 min. As shown in Figure 5A, there was no correlation between the percentage of the peptide that was phosphorylated and the total amount loaded into the cell. If a cell volume of 1 pL is assumed, typical of many mammalian tissue culture cells, the range of peptide concentrations in the cells analyzed in these experiments was 8−600 nM. This concentration range is well below the expected K_M for F-PKB (*4*). At longer incubation times (10 and 30 min), the percentage of PF-PKB remained independent of the number of moles of F-PKB loaded into the cells. For example, in HT1080 cells analyzed 30 min after loading, there was significant overlap in the average percentage of PF-PKB per cell despite greatly different total amounts of intracellular peptide: $1.5-2.5 \times 10^{-21}$ mol, $55 \pm 13\%$ ($n = 4$); $1.4-1.8 \times 10^{-20}$ mol, $64 \pm 7\%$ ($n = 4$); and $7-10 \times 10^{-19}$ mol, $77 \pm 12\%$ ($n = 4$). In addition, comparison of cells analyzed after incubation times of 4, 10, and 30 min revealed that the average percentage of PF-PKB remained essentially unchanged at the different time points (Figure 5B).

To determine whether a simple explanation might exist for the apparent independence of F-PKB phosphorylation on the total concentration of the peptide, the concentrations of F-PKB and PF-PKB were compared to the K_M of the phosphorylation and dephosphorylation reactions. For these experiments, [F-PKB] (6−800 nM) in the cell was well below the K_M (25 μM) of PKB for the parent peptide (*4*). If phosphorylation follows Michaelis−Menten kinetics, then the reaction should exhibit first-order kinetics (since [F-PKB] $\ll K_M$) (*49*). Consequently, the rate of phosphorylation of F-PKB over time will be proportional to [F-PKB]. [PF-PKB]

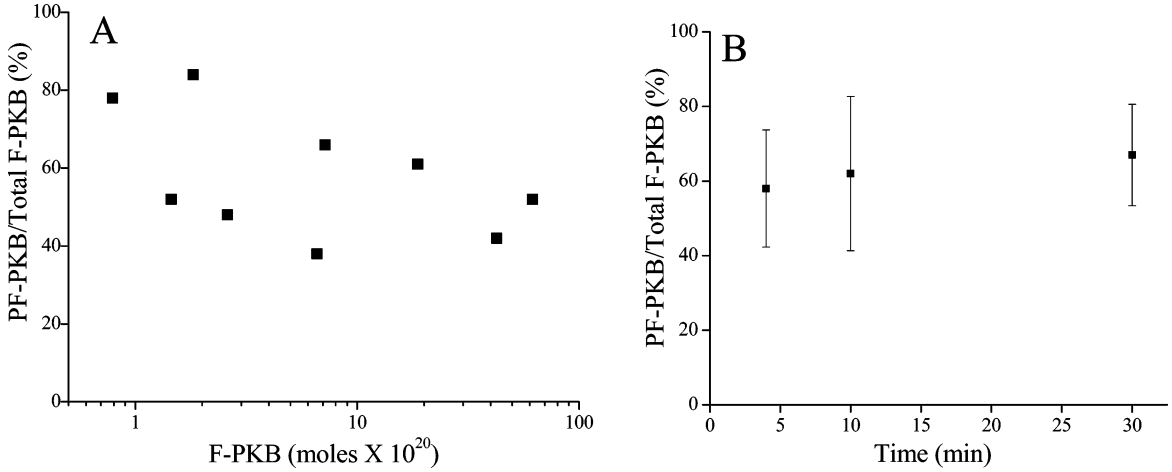

FIGURE 5: Dependence of the phosphorylation of F-PKB on the amount of peptide and incubation time. (A) LMS/CACE was used to determine the total amount of F-PKB loaded per cell and the proportion of the peptide phosphorylated in individual cells. The percentage of PF-PKB in individual HT1080 cells 4 min after microinjection with F-PKB is plotted against the number of moles of F-PKB in each cell. (B) The amount of F-PKB and PF-PKB in HT1080 cells was measured 4, 10, and 30 min (n = 9, 8, and 6 cells, respectively) after F-PKB had been loaded into a cell.

is also predicted to be substantially less than the K_M (typically 10−100 μM for proteins) of PF-PKB for the predominant phosphatases in eukaryotic cells (*50*, *51*). As a result, the rate of dephosphorylation of PF-PKB is expected to be proportional to [PF-PKB]. At a steady state, the rate of phosphorylation must equal the rate of dephosphorylation of the peptide. Consequently, the ratio of [F-PKB] to [PF-PKB] (or to [F-PKB] + [PF-PKB]) is then constant as the total concentration of peptide is varied, so long as the kinetics remain first-order. Under these conditions, the percentage of PF-PKB in the cell at the steady state can be constant despite the different amounts of F-PKB loaded into the cell.

The average percentage of PF-PKB changed very little from 4 min [58 ± 16% (n = 9)] to 30 min [67 ± 14% (n = 14)], although there was significant heterogeneity in the percentage of the peptide phosphorylated among individual cells. We hypothesized that this stable state of peptide phosphorylation was the result of a balance between the activity of PKB and intracellular phosphatases. To test this possibility, we studied the phosphorylation of F-PKB after incubation for 10 min in single HT1080 cells pre-exposed to the phosphatase inhibitor α-NAP. Under these conditions, the percentage of PF-PKB increased from 62 ± 21% (n = 8) to 96 ± 4% (n = 10), demonstrating that all of the loaded peptide was available for phosphorylation. We then compared the amount of substrate peptide and phosphorylated peptide present in the cell when the cell was loaded with either F-PKB or PF-PKB only. After incubation for 4 min, the final percentage of the phosphorylated peptide was similar irrespective of whether the HT1080 cells were loaded with F-PKB [58 ± 16% present as PF-PKB (n = 9)] or PF-PKB [45 ± 18% present as PF-PKB (n = 11)]. Similar results were seen when cells were analyzed after incubation for 2 and 3 min (data not shown). Phosphorylation and/or dephosphorylation of the peptide occurred relatively rapidly to reach a steady state within 2 min. Thus, the assay provided a measure of the balance of catalytic activity between the kinase and phosphatases within the cell.

Introduction of the peptide by microinjection or pinocytic loading could potentially be a stressful stimulus. It was shown

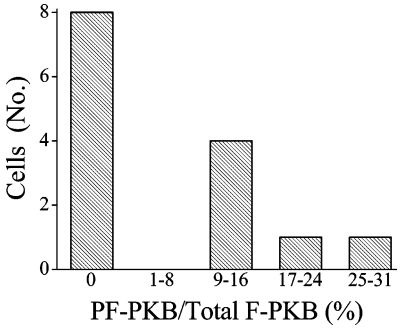

FIGURE 6: Single-cell analysis of HT1080/PTEN cells. Histograms summarizing the results of LMS/CACE analyses of individual HT1080/PTEN cells loaded with F-PKB by microinjection. Cells were allowed to recover for 10 min before analysis.

above that serum-starved NIH-3T3 cells loaded with F-PKB by microinjection displayed little to no PF-PKB. Comparison of the manner in which the HT1080 cells were loaded with peptide showed that the number of moles of F-PKB phosphorylated was similar irrespective of whether the cells were loaded by microinjection [59 ± 19% (n = 6)] or pinocytosis [67 ± 14% (n = 6)]. These findings suggest that these profoundly different approaches to loading the peptide did not contribute significantly to the measurement under these conditions.

Western immunoblot analyses comparing PKB phosphorylated on S^{473} in HT1080 and HT1080/PTEN cells show a reduction, but not an absence, in the level of phospho-S^{473} in the PTEN-expressing cells (*26*). Since this assay measures a population average, two explanations were possible for the low-level, but persistent, active form of PKB. Either all cells in the population possess a small but measurable amount of phosphorylated PKB, or there was a bimodal population of cells differing in activated PKB. To determine the explanation, single-cell assays using F-PKB and the LMS/CACE analysis were used to study the pattern of PKB activity in individual HT1080/PTEN cells. As seen in Figure 6, HT1080/PTEN cells fell into two populations. Most cells (8 of 14) had no detectable PF-PKB, while 6 of 14 cells had an average of 17 ± 8% PF-PKB. In contrast, 100% of the

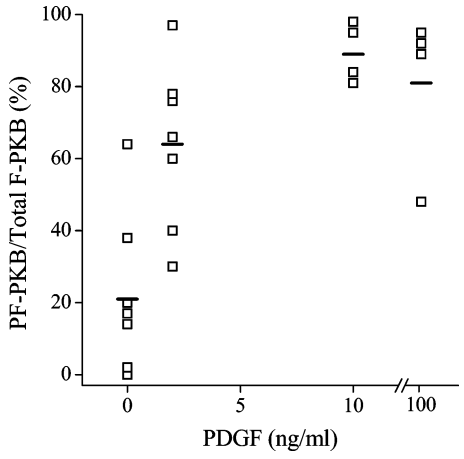

FIGURE 7: Single-cell analysis of NIH-3T3 cells stimulated with PDGF. NIH-3T3 cells were microinjected with F-PKB followed by incubation for 20 min with PDGF. The percentage of PF-PKB in individual cells is plotted against the concentration of PDGF. The bar denotes the mean percentage of PF-PKB from cells analyzed at each concentration of PDGF.

parental HT1080 cells showed high levels of phosphorylated F-PKB (29 of 29 cells). The low level of phospho-S^{473} present in the population of HT1080/PTEN cells was the result of averaging a bimodal population: (1) cells with undetectable PKB activity and (2) cells with diminished, but persistent, PKB activity.

PKB is a downstream target of PI3K in PDGF-stimulated proliferation of NIH-3T3 fibroblasts (*52*). To demonstrate the ability of the peptide to detect physiological PKB activation in response to PDGF-BB, NIH-3T3 cells were stimulated and the percentage of PF-PKB was determined. As seen in Figure 7, in this set of experiments most serum-starved 3T3 cells had some level of PKB activity in the absence of PDGF stimulation, although the average level of phosphorylation was lower than in stimulated cells [0 ng/mL, 21 ± 19% (n = 7)]. The percentage of PF-PKB increased as the PDGF concentration was increased, but reached a plateau at 10 ng/mL [2 ng/mL, 64 ± 23% (n = 7); 10 ng/mL, 89 ± 7% (n = 4); and 100 ng/mL, 81 ± 34% (n = 4)]. These findings are consistent with a known biologically effective concentration range of PDGF-BB (≤10 ng/mL) based on proliferation assays in NIH-3T3 cells (*53*).

DISCUSSION

As peptides become used increasingly in biological investigations, their behavior in the intracellular environment must be understood. This knowledge is particularly important with regard to the use of peptides as substrates and inhibitors of intracellular enzymes. A concern with the use of a short peptide as a kinase substrate is the specificity with which the peptide is phosphorylated by that particular kinase, since such peptides lack the complement of accessory binding domains and tertiary structure of native protein substrates. In the current study, a substrate peptide for PKB was shown to accurately report the activity of the PI3K/PKB pathway in response to physiologic activation, and in cells possessing constitutive activity. These data suggest that peptides based on consensus sequences derived *in vitro* can maintain specificity in the living cell (*1*, *4*). The molecularly engineered HT1080/PTEN cell line provided a particularly effective system for testing the specificity of intracellular peptide phosphorylation. As discussed above, Western immunoblot assays have shown a significant reduction in the level of phosphorylated PKB compared with the parental cell line (*26*). However, a multitude of kinases in other pathways downstream of Ras, including RhoA, Rac1, JNK, and members of the Raf-dependent pathway (Raf/MEK/ERK/Elk-1), remain constitutively active in an apparently PI3K-independent manner at levels equivalent to that of the parental HT1080 cells (*26*). Under these conditions, the PKB substrate peptide was not phosphorylated by the numerous active kinases in these cells. The inability of S6K1, MAPKAP-K1, and PKC blockade to diminish the level of phosphorylation of the peptide lent further support to the fidelity of the phosphorylation of the peptide by PKB in these cells. A substrate peptide similar to that used here has been shown to be phosphorylated by serum- and glucocorticoid-regulated protein kinase (SGK) (*54*). SGK has been shown to be rapidly downregulated under serum-starved conditions and requires several hours to be transcribed after cells have been stimulated with serum (*55*−*57*). Since experiments were performed after serum starvation, it is unlikely that SGK participated in the phosphorylation of the peptide under the conditions used in the current study. In one set of experiments, HT1080 cells were subjected to prolonged exposure to 10% serum prior to analysis. The percentage of PF-PKB was not increased compared to that of serum-starved cells, again suggesting that under these conditions phosphorylation of F-PKB by SGK was not significant. Further analysis will be required, however, to confirm that F-PKB does not serve as a substrate for SGK in cells.

Although the average steady-state level of F-PKB phosphorylation was consistent under identical experimental conditions, there was significant heterogeneity in the percentage of the peptide phosphorylated among individual cells. The degree of variation seen was similar to the heterogeneity in individual cell responses typically identified in measurements of intracellular calcium concentration and gene expression following cellular stimulation (*58*, *59*). Below the K_M of the peptide for PKB, the amount of F-PKB loaded into the cell and the time of incubation had no appreciable effect on the degree of heterogeneity after steady state conditions were reached. This finding may be explained if both the kinase and phosphatase reactions follow first-order kinetics. In all cells exposed to the phosphatase inhibitor, virtually complete phosphorylation of the peptide was seen; therefore, the heterogeneity in the absence of the inhibitor cannot be explained by variable amounts of sequestration limiting phosphorylation of the peptide within the cell. It is probable that individual cell differences in protein expression and the level of activity in signal transduction pathways involving PKB resulted in the cell-to-cell differences seen here. In the HT1080 cells, these differences may be in part related to analysis of individual cells in different stages of the cell cycle, since HT1080 cells continue through the cell cycle even during exposure to reduced serum concentrations.

There are now a number of approaches to assaying PKB in single cells which are expected to yield important new insights into cell physiology (*60*). Recently, in a manner analogous to Western assays, investigators used fluorescently tagged, phospho-specific antibodies to label PKB phosphorylated at S^{473} in fixed cells followed by flow cytometric

analysis (*61*). This assay provides a means for high-throughput analysis of the phosphorylated form of PKB in single nonadherent cells, but requires fixation and permeabilization of cells prior to labeling, and like the Western assays on which it is based, the method is an indirect measure of catalytic potential. Green fluorescent protein fusions with full-length PKB or its pleckstrin homology domain have been used to follow the dynamics of the translocation of PKB from the cytosol to the plasma membrane after cell stimulation (*62*−*64*). However, these translocation assays examine the presence of PIP_3 generated by PI3K rather than PKB activity, as PKB can remain catalytically inactive after recruitment to the membrane (*65*, *66*). Recently, an indicator of PKB activity based on fluorescence resonance energy transfer (FRET) has been reported (*67*). While the advantages of FRET-based indicators include subcellular imaging and measurements made in real time, a number of caveats must be kept in mind (*60*). The GFP-based indicators frequently display poor signal-to-noise ratios, display unexpected behaviors, and may require expression at levels that compete with native substrates. The microanalytic assay used in the current paper provides a complementary approach to these single-cell assays of PKB by providing quantitative measurements of activity at discrete time points. To date, the authors have utilized this method to assay PKB activity in a number of cell types, including TF-1, K562, and peripheral blood cells from patients with chronic myelogenous leukemia, demonstrating its broad applicability (*68*).

The value of this peptide-based, single-cell assay was readily demonstrated in the analysis of the heterogeneity of PKB activity in HT1080/PTEN cells. It was known from Western immunoblot analyses of phospho-S^{473} in HT1080/PTEN cell lysates that the levels of phosphorylated PKB were significantly reduced compared with that of the parental cell line; nevertheless, a low level of activity persisted (*26*). Population-based, semiquantitative assays such as Western assays cannot determine if this finding was the result of all cells possessing a small, but measurable, level of PKB activity or was due to a bimodal population of cells differing in PKB activity. We have shown in this instance that the reduced level of phospho-S^{473} in the Western immunoblot analysis was the result of the presence of two populations of cells with and without catalytically active PKB. This example shows that the determination of single-cell heterogeneity is required to fully understand the results of assays yielding a population-averaged measurement. Such data are valuable for biochemical studies in which the heterogeneous behavior of cells is critical to the understanding of the biological phenomena being studied.

A further example of the power of this peptide-based assay was provided by performing quantitative measurements of peptide phosphorylation in response to the physiologic stimulus PDGF. Interestingly, while some unstimulated, serum-starved, NIH-3T3 cells were devoid of PKB activity, most had detectable PF-PKB. This finding is consistent with data obtained from *in vitro* kinase assays that show a baseline level of PKB activity under similar conditions (*52*). NIH-3T3 cells were analyzed on two separate occasions during this study. In the second set of experiments, the percentage of PF-PKB in serum-starved cells was increased relative to that of the first set of experiments. Since these experiments were performed on cells thawed at different times, it is likely that the higher percentage of PF-PKB in the second batch of cells represents a higher percentage of spontaneously transformed cells (*69*). Despite this baseline activity and the single-cell heterogeneity, an increase in the average PF-PKB concentration could be easily detected in a dose−response curve at increasing concentrations of PDGF. This increase reached a plateau at the upper limit of PDGF's expected biological range. These experiments demonstrate a valuable advantage to the use of peptides as intracellular reporters. The change in activity of a native kinase in response to a physiological stimulus can be determined without the need for molecular engineering; thus, primary cells can be analyzed by this approach. In addition, the chemical separation step provides the ability to assess the activation of PKB in combination with that of other kinases simply by loading additional kinase reporters into the cell (*38*).

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