Published in final edited form as:
Nat Biotechnol. 2009 October ; 27(10): 933–940. doi:10.1038/nbt.1566.

Sensitive multiplexed analysis of kinase activities and activity-based kinase identification

Kazuishi Kubota[1,7,8], **Rana Anjum**[1,7,8], **Yonghao Yu**[1], **Ryan C Kunz**[1], **Jannik N Andersen**[2], **Manfred Kraus**[2], **Heike Keilhack**[2], **Kumiko Nagashima**[2], **Stefan Krauss**[2], **Cloud Paweletz**[2], **Ronald C Hendrickson**[3], **Adam S Feldman**[4], **Chin-Lee Wu**[5], **John Rush**[6], **Judit Villén**[1], and **Steven P Gygi**[1]

[1] Department of Cell Biology, Harvard Medical School, Boston, Massachusetts, USA

[2] Merck Research Laboratories, Boston, Massachusetts, USA

[3] Merck & Co., Inc., Rahway, New Jersey, USA

[4] Department of Urology, Massachusetts General Hospital, Boston, Massachusetts, USA

[5] Department of Pathology, Massachusetts General Hospital, Boston, Massachusetts, USA

[6] Cell Signaling Technology, Danvers, Massachusetts, USA

Abstract

Constitutive activation of one or more kinase signaling pathways is a hallmark of many cancers. Here we extend the previously described mass spectrometry–based KAYAK approach by monitoring kinase activities from multiple signaling pathways simultaneously. This improved single-reaction strategy, which quantifies the phosphorylation of 90 synthetic peptides in a single mass spectrometry run, is compatible with nanogram to microgram amounts of cell lysate. Furthermore, the approach enhances kinase monospecificity through substrate competition effects, faithfully reporting the signatures of many signaling pathways after mitogen stimulation or of basal pathway activation differences across a panel of well-studied cancer cell lines. Hierarchical clustering of activities from related experiments groups peptides phosphorylated by similar kinases together and, when combined with pathway alteration using pharmacological inhibitors, distinguishes underlying differences in potency, off-target effects and genetic backgrounds. Finally, we introduce a strategy to identify the kinase, and even associated protein complex members, responsible for phosphorylation events of interest.

Correspondence should be addressed to S.P.G. (steven_gygi@hms.harvard.edu).
[7]Present addresses: Daiichi Sankyo Co., Ltd., Tokyo, Japan (K.K.) and ARIAD Pharmaceuticals Inc., Cambridge, Massachusetts, USA (R.A.).
[8]These authors contributed equally to this work.

Accession code. Raw data files corresponding to the data shown in Figure 3 are available at the Tranche website (https://proteomecommons.org/tranche/about.jsp).

Note: Supplementary information is available on the Nature Biotechnology website.

AUTHOR CONTRIBUTIONS

K.K. and R.A. are co-first authors. K.K., R.A., Y.Y., R.C.K., J.V. and S.P.G. participated in the planning, data generation and data interpretation. J.N.A., M.K., H.K., K.N., S.K., C.P. and R.C.H. prepared cell lysates using Akt and PDK1 inhibitors. A.S.F. and C.-L.W. prepared human renal carcinoma samples. J.R. synthesized all peptides. K.K. and S.P.G. wrote the manuscript and all authors edited it.

Most cellular signaling pathways are regulated by post-translational modification of proteins, with phosphorylation being among the most prominent. Overexpression or constitutive activation of a receptor tyrosine kinase (RTK) is often a transformative event in oncogenesis[1,2]. In addition, RTK-independent activation of the phosphatidylinositol 3-kinase (PI3K) pathway (e.g., by loss of phosphatase and tensin homolog (PTEN) protein function) and mitogen-activated protein kinase (MAPK) pathway (e.g., by gain of K-Ras function) are two of the most frequently observed causes of human malignancy. With several approved small molecule kinase inhibitors and >200 others in development, rapid and accurate assessment of the activation states of oncogenic kinase pathways in different tumors will be crucial to making appropriate therapy decisions in personalized medicine.

Most strategies to infer kinase activities involve using phospho-specific antibodies to monitor phosphorylation of specific residues in protein kinases or their substrates. Although phosphorylation at a particular site provides a helpful surrogate for the kinase activation state, it is only an indirect measurement of kinase activity. Such methods are thus often viewed as qualitative or, at best, semiquantitative. On the other hand, strategies designed to measure direct phosphorylation rates include arrays of ~1,000 peptides on glass slides[3,4], a multiplexed kinase assay to simultaneously measure four kinase activities[5] and a solution-phase phosphorylation reaction with 900 peptide-oligonucleotide substrates[6]. However, most array-based approaches are unable to resolve the actual site of substrate phosphorylation, which is important for minimizing off-target events. Moreover, use of unpurified peptides reduces the confidence in quantification accuracy. Therefore, highly quantitative and direct measurement methods that simultaneously report activities of many phosphorylation pathways are still needed to address the diverse clinical manifestations of signaling in cancer and to choose optimal treatments.

Due to its specificity and precise quantitative nature, mass spectrometry (MS) represents an ideal platform to quantify products formed from enzymatic reactions[7,8]. Recently, we presented the KAYAK (kinase activity assay for kinome profiling) method[9] for the multiplexed measurement of 90 different peptide phosphorylation rates from cell lysates. In this approach, each peptide, which carries a known phosphorylation site, is phosphorylated separately in a 96-well plate format. Otherwise identical stable isotope–labeled phosphopeptides are then added to provide absolute quantification of site-specific phosphorylation rates during liquid chromatography (LC)-MS analysis. The reliance on 90 separate kinase reactions makes consumption of substantial amounts of lysate a limitation of this assay. Moreover, it provides limited knowledge of kinase-peptide specificities.

Here we improve this methodology to enhance throughput and multiplicity by assessing phosphorylation rates for all 90 of the same peptides used previously (ref. 9) in a single reaction. The modified KAYAK strategy faithfully reports the activation of cellular signaling pathways including mitogen stimulation of HEK-293 and HeLa cells. Furthermore, combining single-reaction KAYAK with kinase inhibition enables pathway specificity to be resolved. For example, we identified cAMP-dependent protein kinase (PKA) pathway hyperactivation in MCF7 cells and cell line-specific cross-talk between the PI3K and MAPK pathways in HEK-293 but not HeLa cells. Moreover, in conjunction with deep protein sequencing and correlation profiling of separated lysates, we demonstrate that a KAYAK-based strategy can identify direct kinase-substrate pairs and even regulatory partners in kinase-containing complexes.

RESULTS

Development and validation of a single-reaction KAYAK assay

Our scheme for 90 simultaneous kinase-activity measurements is illustrated in Figure 1, with a detailed description provided in Supplementary Note. Briefly, we chose substrate peptides to represent several core signaling pathways as well as protein sites identified by large-scale phosphoproteomics studies[10,11] but with no associated kinase (Supplementary Table 1). *In vitro* kinase assays were performed in a single reaction containing the kinase source (typically cell lysate) and the mixture of the 90 KAYAK peptides used previously[9]. After quenching the reactions with acid, we added 90 stable isotope–labeled internal standard phosphopeptides, enriched the phosphorylated peptides using immobilized metal-ion affinity chromatography (IMAC) and then analyzed them by LC-MS. As a known amount of each heavy phosphopeptide was added, the ratio of the light to the heavy phosphopeptide provided the absolute amount of each product formed during the reaction. A major difference from the previous study[9] was that reacting the substrate peptides as a mixture rather than individually enabled notably higher throughput and 90-fold less sample consumption in a reaction volume of only 50 μl. The tendency of many kinases to phosphorylate similar peptides complicates the use of multiplexed kinase assays to match individual kinase activities in crude extracts with their endogenous substrates. To reduce cross-phosphorylation of peptides by different kinases, we decreased the concentration of each peptide from 100 μM to 5 μM. We then profiled the entire library using 20 commercially available purified kinases to assess candidate kinases for each peptide (Supplementary Fig. 1). Although purified kinases are known to be more promiscuous than when unpurified[12], this provided a preliminary assessment of potential kinases that can be matched to each substrate and the degree of monospecificity for each peptide.

We benchmarked assay performance using lysate from a transformed human epithelial cell line (HEK-293) after insulin stimulation (Fig. 2a). The sensitivity of each peptide was assessed using lysate amounts varying from 1 ng to 20 μg. Phosphorylation of at least half of the library was measureable with site-specific phosphorylation of >50 fmol using 10 or 20 μg of lysate. Notably, eight peptides were phosphorylated from 10 ng lysate, which corresponds to ~20 cells, and two exceptional peptides were phosphorylated using only 1 ng lysate (Supplementary Fig. 2b). Most importantly, the vast majority of peptides (88%, 43 peptides among 49 peptides detected at more than one concentration) demonstrated a linear response to lysate amount ($r > 0.9$), suggesting that lysate amount (or sample dilution) is not a limiting factor in kinase activity measurements (Fig. 2b and Supplementary Fig. 2a). We next found excellent assay reproducibility across five dishes of HEK-293 cells and across three insulin stimulation experiments (Supplementary Figs. 3 and 4 and Supplementary Note).

When we used lysates from cells before and after insulin stimulation to compare this improved KAYAK single-reaction strategy with the earlier strategy of performing 90 individual kinase reactions in a plate format under identical conditions, we observed excellent agreement between both reaction strategies (Fig. 2c). Only three peptides (A3, E11 and F6) showed reproducibly greater phosphorylation in response to insulin stimulation. Performing the assay in a single reaction resulted in more robust changes for each peptide compared to the individual reaction method, likely because competitive effects widened the gap between the best and alternative kinase substrates[13]. Likewise, use of single-reaction KAYAK to profile epidermal growth factor (EGF) stimulation in HeLa cells demonstrated advantages over the individual reaction method, which we also attribute to competition effects (Supplementary Fig. 5).

Insulin and EGF treatment show distinct activity profiles

To distinguish basal cellular kinase activity from stimulated states, we compared kinase activities from serum-starved HeLa and HEK-293 cells treated with insulin, EGF or phorbol 12-myristate 13-acetate (PMA) using the same 90 peptides used previously[9] (Fig. 3). After hierarchical clustering of the normalized activities, peptides preferentially phosphorylated by a particular kinase in an *in vitro* assay using purified enzyme (Supplementary Fig. 1) clustered together. Compared to HeLa cells, HEK-293 cells were twice as responsive to insulin stimulation as measured by the A3 peptide (Fig. 3b), a highly selective substrate of Akt[14]. This was confirmed by western blot analysis (Fig. 3c). In addition, the E11 peptide, which has a 90-kDa ribosomal S6 kinase (RSK) phosphorylation motif[15] and is preferably phosphorylated by purified RSK1 enzyme (Supplementary Fig. 1), displayed greater phosphorylation after activation of Ras/MAPK pathway by EGF or PMA treatment (Fig. 3b). Again, this is consistent with western blot analysis (Fig. 3c; and Supplementary Notes for a detailed discussion of E11 specificity). As the KAYAK methodology measures the absolute amount of phosphorylated peptides, the observed difference in basal kinase activities between HEK-293 and HeLa cells toward the E11 peptide likely reflects differences in kinase activity states, which we also observed using western blotting. Overall, although the basal levels and fold changes in kinase activities were often different in these two cell lines, the direction of change for each peptide in response to each stimulus was consistent (Supplementary Fig. 6), highlighting conserved signaling pathways.

Basal kinase activity profiles of 11 human cell lines

Baseline profiling of kinase activation state can identify aberrantly activated pathways and cellular processes. To identify unique signatures, we profiled kinase activities from 11 human cell lines grown under recommended conditions (Fig. 4 and Supplementary Fig. 7). Hierarchical clustering grouped peptides with similar activity profiles across the cell lines (Fig. 4a), identifying marked differences in core pathway activation states. The MCF7 breast cancer cell line, for example, demonstrated uniquely and surprisingly high levels of PKA activity (Fig. 4b), which was confirmed by a specific PKA inhibitor peptide (Supplementary Fig. 8). This observation is consistent with a report that compared normal (MCF10A) and tumor (MCF7) cell lines[16]. The U-87 MG glioblastoma cell line had between 3- and 20-fold higher basal phosphorylation of the Akt-selective peptide, A3, compared to any other cell line in the panel. U-87 MG bears a frameshift mutation in PTEN[17], which leads to elevated phosphatidylinositol 3,4,5-triphosphate levels and hyperactivation of Akt. The PTEN-deficient Jurkat T-lymphocyte cell line[18] also showed high A3 phosphorylation, which was confirmed by western blot analysis (Fig. 4c). Moreover, Jurkat cells displayed upregulated Tyr kinase activities and the tyrosine-phosphorylated peptides clustered into at least three different groups (Fig. 4a,b), demonstrating the detection of multiple activated tyrosine kinase pathways. Agreement between kinase activity and gene mutation in the Ras/MAPK pathway is discussed further in Supplementary Note. We plan to obtain activity profile signatures for many hundreds of cell lines to both highlight the pathways most commonly mutated and predict appropriate pharmacological intervention. In these 11 cell lines, KAYAK profiling clearly demonstrated phosphorylation events and patterns specific to each cell line. Although we have only a general biological association for each cluster, the unique kinase activity signature for an individual cell line reflects key differences in either pathway activation or regulation.

Our interest in the PI3K pathway and cancer next led us to apply our single-reaction KAYAK strategy to examine a genetically modified mouse model in which KAYAK profiling reported the individual and net signaling effect of PTEN knockout and/or 3-phosphoinositide–dependent protein kinase-1 (PDK1) knockdown, respectively (Supplementary Fig. 9 and Supplementary Note). Whereas PTEN deletion increased basal

Akt activation, PDK1 knockdown did not. In contrast, PKC-targeted peptide phosphorylation was selectively reduced by PDK1 knock down, but not by PTEN deletion.

To further explore kinase profiling in a clinical environment, we profiled human renal carcinoma patient specimens after radical nephrectomy. Matched tumor and control tissue were taken from five affected kidneys (Supplementary Fig. 10 and Supplementary Note). All five tumor samples had greater PI3K activity than the control, as measured by activated Akt. However, RSK/extracellular signal-regulated kinase (ERK) pathway activation in tumor lysates varied, and patient number 4 demonstrated extremely high activities for both PKA and tyrosine kinases. We conclude that KAYAK profiling can be extended to clinical specimens and could thus aid patient-specific tailoring of kinase inhibitor therapies.

Analysis of kinase-inhibitor effects by KAYAK

Predicting the cellular effects of kinase inhibitors is challenging despite design efforts to achieve selective inhibition of a single target[2,19]. To evaluate the activity profiles of commonly used kinase inhibitors, we treated HEK-293 cells with various reference compounds, and followed this with insulin stimulation and single-reaction KAYAK analysis using all 90 peptides (Supplementary Fig. 11). This pharmacological study conclusively demonstrated that insulin-dependent activation of E11 was achieved through cross-talk of the PI3K and MAPK pathways in HEK-293 cells (Supplementary Note). This cross-talk is a cell line–specific effect that does not occur in, for example, HeLa cells (Fig. 3b).

Due to the central role of the PI3K/Akt pathway in tumorigenesis, inhibitors of Akt may be useful in cancers that depend on PTEN loss or oncogenic PI3K mutations for survival[20,21]. By the same token, PDK1 is being pursued as an anticancer drug target as it regulates the activity of numerous oncogenic kinases, including Akt, p70S6K and RSK[22]. To explore the potential of the single-reaction KAYAK strategy for use in drug discovery, we evaluated the activities of structurally diverse kinase inhibitors in cancer cell lines with deregulated PI3K pathway activity. Inhibitors were selected from the literature[22] and identified from a focused kinase library screen[23,24] to have affinity for PDK1 (Supplementary Fig. 12a). For comparison, a highly selective and allosteric Akt inhibitor[25] was included.

Single-reaction KAYAK profiling identified profound differences in baseline pathway activities of the selected cancer cell lines, including two PTEN null prostate cancer cell lines, PC-3 and LNCaP (Fig. 5a). Hierarchical clustering of the 90 peptides, which groups activities into kinase clusters (e.g., PKA, PKC and pTyr), revealed that PC-3 cells maintain approximately tenfold higher PKC and RSK/ERK pathway activities than LNCaP cells (Fig. 5b; DMSO). In contrast, basal Akt activity, as measured by phosphorylation of the A3 peptide, was similar for all three cell lines (Fig. 5b; DMSO). Thus, the A2780 ovarian cancer cell line, which expresses an activating PI3K mutation (E365K mutation of p110α) and a truncated PTEN protein (K128-R130 deletion), has elevated Akt activity levels comparable to the two PTEN null prostate cancer cell lines (Fig. 5; LNCaP and PC3) and the PTEN-deficient U-87 MG and Jurkat cells (Fig. 4b). This elevated Akt activity is tenfold higher than in cancer cell lines expressing wild-type PTEN (Fig. 4b) and highlights the large dynamic range and quantitative nature of the assay for cellular profiling studies.

Notably, PDK1 inhibitors were more effective at reducing Akt activity in PC-3 and A2780 cells than LNCaP cells (Fig. 5b). This result is in accordance with western blot analysis (Supplementary Fig. 12c). By contrast, the allosteric Akt inhibitor potently inhibited A3 phosphorylation in all cell lines tested (Fig. 5b). Because PDK1 also phosphorylates the activation domain of RSK[15], PDK1 inhibitors (but not the Akt inhibitor) greatly reduced RSK/ERK pathway activity as measured by the E11 peptide (Fig. 5b), although there were potency differences. Western blot analysis confirmed the inhibition of RSK in cell lines

treated with PDK1 inhibitors, with PDK1i-3 being the least potent (Supplementary Fig. 12c). Indeed, hierarchical clustering further highlights these differences (Fig. 5c). In LNCaP cells, for example, the Akt inhibitor did not cluster with any treatments as it was uniquely potent at inhibiting Akt activity compared to the PDK1 inhibitors, which clustered together. On the other hand, in PC-3 cells, the Akt inhibitor clustered with the two most potent and structurally similar PDK1 inhibitors (PDK1i-2 and PDK1i-4) consistent with potent inhibition of Akt in this cell line (Fig. 5c and Supplementary Fig. 12a). Interestingly, one compound (PDK1i-1) showed strong inhibition of multiple pathways, including the PKA and PKC kinase clusters. We conclude that KAYAK profiling can indicate the cellular specificity of kinase inhibitors, guiding lead optimization of selective compounds targeting a specific pathway.

An activity-based kinase identification strategy

It is often highly desirable to identify the kinase responsible for a particular phosphorylation event. Although purified kinases provide a good starting point (Supplementary Fig. 1), testing the ~500 kinases in the human genome[26] is not practical and also fails to capture the cellular context of these enzymes. To address this issue, we developed a biochemical strategy to use single-reaction KAYAK profiling to identify the kinase responsible for phosphorylating a peptide substrate. A lysate of interest is first fractionated by column chromatography (Supplementary Fig. 13), and an activity profile for each fraction is determined. In parallel, an aliquot of each fraction is digested with trypsin and analyzed by LC-tandem MS (MS/MS) to identify and assess the abundance of thousands of proteins, providing a protein profile for each fraction. Correlation of the activity and kinase abundance profiles across active fractions is then used to pinpoint the responsible kinase.

We elected to validate the methodology by identifying a mitotic kinase activity from HeLa cells. Figure 6a shows a heat map of the kinase activities from three different HeLa cell lysates: asynchronous, G1/S-phase arrested, or G2/M-phase arrested. Hierarchical clustering revealed differences in many activities including some mitosis-specific ones. Seven peptides sharing a common motif of [S/T]-Pro and clear upregulation by G2/M arrest (Fig. 6b and Supplementary Fig. 14a) were selected for correlation profiling experiments to identify the responsible kinase. We separated lysate from nocodazole-arrested HeLa cells by high-resolution anion exchange chromatography, and collected the flow-through and 36 fractions (Fig. 6c) before assessing the activity profile for each peptide (Fig. 6d). Strikingly, all seven peptides demonstrated the identical pattern of normalized phosphorylation rates, suggesting that a single kinase was responsible for their phosphorylation. Trypsin digestion and shotgun sequencing of each fraction by LC-MS/MS identified 3,933 proteins, including 114 kinases (Supplementary Fig. 14c). The correlation profile for all proteins was assessed based on normalized spectral counting (Online Methods). Calculating the Pearson correlation coefficient between kinase activity and protein amount in the active fractions (Supplementary Fig. 15), we found Cdc2 as the best-ranked kinase and 8th overall among 3,933 proteins (Fig. 6e and Supplementary Fig. 16). Protein quantification of Cdc2 showed two major peaks with the second correlating with the kinase activity profile (Fig. 6e). Notably, this second peak also showed an excellent correlation profile with Cyclin B1, which ranked 5th overall among all proteins and is required for Cdc2 activity[27,28]. Western blot analysis confirmed the MS-based results (Supplementary Fig. 17). Moreover, purified Cdc2/Cyclin B1 complex phosphorylated all seven peptides along with four other upregulated peptides (Fig. 6f). Finally, immunodepletion using anti-Cyclin B1 removed >88% of kinase activity for all seven upregulated peptides (Supplementary Fig. 18). These experiments identified Cdc2 as the most likely kinase and Cyclin B1 as an active complex member for the phosphorylation of these seven peptides. One peptide (A6) is predicted to be an ERK and p38 MAPK target using Scansite[29].

As a further example, we applied activity-based correlation profiling to identify two additional activities from Jurkat cells. PRKAA1 (also known as AMPK1) phosphorylated E5 in Jurkat cell lysates (Supplementary Fig. 19), and the tyrosine kinase Lck phosphorylated D11 (Supplementary Fig. 20 and Supplementary Note).

DISCUSSION

Chemically synthesized peptides of optimized sequence have been used for more than 30 years as *in vitro* phosphorylation substrates using both purified kinases and cell lysates[30–32]. Our single-reaction KAYAK strategy simultaneously measures 90 peptide-based phosphorylation rates using high-resolution MS. We selected substrate peptides from optimized targets or from uncharacterized sites on interesting proteins[9] to encompass diverse signaling pathways and enhanced measurement accuracy by using stable isotope–labeled internal standards. The purified peptides used in single-reaction KAYAK profiling ensure absolute quantification of activities that are linear over several logs of lysate amounts. The approach also partially addresses the kinase-specificity problem inevitably associated with peptide-based measurements (Supplementary Note). Finally, based on the many cellular settings investigated, the assay appears to faithfully and simultaneously report the core activation states for many pathways, including the PI3K and MAPK pathways, which are most frequently altered in cancer.

Compared to other strategies, the KAYAK strategy[9] has several advantages. First, KAYAK directly monitors kinase enzymatic activities, thereby averting the need for an activity-indicating antibody. Although commonly used, phosphorylation-activity relationships are known to be far from ideal. Moreover, activation-state phospho-antibodies are not available for many kinases. Second, single-reaction KAYAK measures the intrinsic activity of multiple kinases reflecting the complex cellular context. Although high-throughput kinase assays using large kinase panels are becoming feasible[33], those approaches often use truncated or recombinant purified enzymes, which may not reflect the actual conformational or kinase activity state as they appear in cells. Third, KAYAK has high sensitivity owing to the signal-amplifying nature of enzymatic reactions. Using our single-reaction KAYAK strategy with 20-fold lower peptide concentrations than used previously[9], two KAYAK peptides showed detectable phosphorylation from as little as 1 ng of cell lysate, which corresponds near single-cell levels (Fig. 2a and Supplementary Fig. 2). The sensitivity of the single-reaction KAYAK approach permits very low sample consumption. Practically, 10–20 μg of cell lysate produce reliable signals for about 50 simultaneous peptide reactions (Fig. 2a). Fourth, the approach measures site-specific phosphorylation rates. Commonly, additional phosphorylatable residues are available[34]. As the internal standard peptides were synthesized with phosphorylation at known positions, the co-elution of lysate-phosphorylated peptides and the standard phosphopeptides in conjunction with fragmentation sequencing ensures that site-specific phosphorylation is measured. This is not accomplished by any alternative method. Fifth, single-reaction KAYAK is highly quantitative, with exceptional reproducibility (Supplementary Figs. 3 and 4). Internal standards of heavy peptides, which are added upon quenching the kinase reaction, cancel any downstream sample manipulation and measurement variations and provide the basis for absolute activity measurements (that is, fmol phosphorylation/μg lysate/min). Western blot analysis does not offer a similar level of quantitative quality. Sixth, the assay and protocol can be applied across a wide range of cellular settings, including recombinant purified enzymes (Supplementary Fig. 1), cell line lysates (Figs. 3–5), mouse primary tissue cultures (Supplementary Fig. 9) and clinical tissue samples (Supplementary Fig. 10). Seventh, KAYAK is radio-isotope free. Finally, single- reaction KAYAK is flexible in terms of substrate concentration and peptide number. We believe that the single-reaction KAYAK protocol could exploit >90 peptides by using shallower gradients during LC-MS analyses.

However, our current focus is to remove redundant or less useful peptides while adding peptides that are monospecific for underreported kinases and pathways.

Single-reaction KAYAK is not without disadvantages. As it is based on an *in vitro* kinase assay, the current implementation does not measure kinase activities within an intact cell. Moreover, owing to its peptide-based nature, the activity of a kinase that requires a conformational substrate may not be suitably monitored. Finally, the specificities of many kinases toward a peptide substrate may not be unique even at the 5 μM level. Therefore, some peptides may still be phosphorylated by more than one kinase, which complicates pathway identification.

Although *in vitro* phosphorylation using purified kinases (Supplementary Fig. 1) cataloged likely kinase candidates for many phosphorylation events, identification of the responsible kinase directly from cell lysates confirms the physiological relevance of the kinase-substrate pairs. Whereas identifying a phosphorylation event using a specific kinase is relatively straightforward[35], developing a general methodology to identify a kinase responsible for a specific phosphorylation event is far more challenging[36–39]. A series of chemical reagents that can cross-link a kinase and its substrate may meet this need, but the reagents have not been shown to work in complex situations, such as those involving cell lysates[40,41]. Although traditionally, identification of a responsible enzyme for a specific activity has been accomplished by comparing enzymatic activity and a protein band after SDS-PAGE gel separation, this correlation requires multiple purification steps. Owing to the advancement of protein quantification by MS[42], correlation profiles have been used to determine protein localization by MS[43–45]. This has renewed interest in applying modern quantitative proteomics using the classic concept of comparing enzyme activities and protein profiles. Our strategy is a general methodology to decipher kinase-substrate relationships starting with a phosphorylated peptide substrate and a minimally fractionated lysate.

Phosphoproteomics projects have delivered atlases of experimentally mapped phosphorylation sites[10,11,46–50]. Yet, many phosphorylation sites and/or motifs have not been associated with a kinase and may be referred to as “orphan”[41]. Indeed, during this proof-of-concept study, we found that one unpredicted peptide was phosphorylated by Cdc2/Cyclin B1 complex in a specific cellular context. Although a fraction of these sites may be phosphorylated in the context of the appropriate three-dimensional protein fold, most would be expected to be phosphorylated with a high degree of specificity owing to primary sequence determinants. We predict that the combination of activity profiles and protein correlation profiling will bridge the gap between large-scale phosphoproteomics efforts to characterize phosphorylation events and better insight into their biological context and function.

Although considerable challenges remain, the single-reaction KAYAK strategy has the potential to impact kinase-based therapies on many levels. KAYAK profiles might influence a lead compound decision at an early phase by revealing off-target effects or potency differences in highly related molecules or suggest a tractable biomarker of drug efficacy needed in clinical trials. Finally, single-reaction KAYAK profiles could enable physicians to tailor drug choice or dosing to address the aberrant signaling events underlying a patient’s particular pathology.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturebiotechnology/.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by grants from the National Institutes of Health (NIH; HG3456 and GM67945) and an industry-sponsored research project to S.P.G. from Merck and ThermoFisher. K.K. was supported by Daiichi Sankyo Co., Ltd. Y.Y. was partly supported by J. Blenis through grants from the NIH (GM51405). J.V. was supported by a grant from the Spanish Ministry of Education and Science. We thank C. Zhou, S. Elledge and N. Dephoure for help with cell cycle sample preparation, J. Elias and D. Kolippakkam for bioinformatics support, M. Rodriguez-Falcon for establishing Stagetip-IMAC protocol, and S. Sando and T. Robbins for peptide purification. We appreciate the advice and encouragement of many members of the Gygi lab and Mayumi Kubota.

References

1. Krause DS, Van Etten RA. Tyrosine kinases as targets for cancer therapy. N Engl J Med. 2005; 353:172–187. [PubMed: 16014887]
2. Sebolt-Leopold JS, English JM. Mechanisms of drug inhibition of signalling molecules. Nature. 2006; 441:457–462. [PubMed: 16724058]
3. Diks SH, et al. Kinome profiling for studying lipopolysaccharide signal transduction in human peripheral blood mononuclear cells. J Biol Chem. 2004; 279:49206–49213. [PubMed: 15355981]
4. Houseman BT, Huh JH, Kron SJ, Mrksich M. Peptide chips for the quantitative evaluation of protein kinase activity. Nat Biotechnol. 2002; 20:270–274. [PubMed: 11875428]
5. Janes KA, et al. A high-throughput quantitative multiplex kinase assay for monitoring information flow in signaling networks: application to sepsis-apoptosis. Mol Cell Proteomics. 2003; 2:463–473. [PubMed: 12832460]
6. Shults MD, et al. A multiplexed protein kinase assay. Chem Bio Chem. 2007; 8:933–942.
7. Gao H, Leary JA. Multiplex inhibitor screening and kinetic constant determinations for yeast hexokinase using mass spectrometry based assays. J Am Soc Mass Spectrom. 2003; 14:173–181. [PubMed: 12648923]
8. Pi N, Armstrong JI, Bertozzi CR, Leary JA. Kinetic analysis of NodST sulfotransferase using an electrospray ionization mass spectrometry assay. Biochemistry. 2002; 41:13283–13288. [PubMed: 12403630]
9. Yu Y, et al. A site-specific, multiplexed kinase activity assay using stable isotope dilution theory and high-resolution mass spectrometry. Proc Natl Acad Sci USA. 2009; 106:11606–11611. [PubMed: 19564600]
10. Beausoleil SA, et al. Large-scale characterization of HeLa cell nuclear phosphoproteins. Proc Natl Acad Sci USA. 2004; 101:12130–12135. [PubMed: 15302935]
11. Villen J, Beausoleil SA, Gerber SA, Gygi SP. Large-scale phosphorylation analysis of mouse liver. Proc Natl Acad Sci USA. 2007; 104:1488–1493. [PubMed: 17242355]
12. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell. 2007; 129:1261–1274. [PubMed: 17604717]
13. Ubersax JA, Ferrell JEJ. Mechanisms of specificity in protein phosphorylation. Nat Rev Mol Cell Biol. 2007; 8:530–541. [PubMed: 17585314]
14. Alessi DR, Caudwell FB, Andjelkovic M, Hemmings BA, Cohen P. Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. FEBS Lett. 1996; 399:333–338. [PubMed: 8985174]
15. Anjum R, Blenis J. The RSK family of kinases: emerging roles in cellular signalling. Nat Rev Mol Cell Biol. 2008; 9:747–758. [PubMed: 18813292]
16. Sigoillot FD, Sigoillot SM, Guy HI. Breakdown of the regulatory control of pyrimidine biosynthesis in human breast cancer cells. Int J Cancer. 2004; 109:491–498. [PubMed: 14991569]
17. Chou TF, et al. 31P NMR and genetic analysis establish hinT as the only Escherchia coli purine nucleoside phosphoramidase and as essential for growth under high salt conditions. J Biol Chem. 2005; 280:15356–15361. [PubMed: 15703176]

18. Astoul E, Edmunds C, Cantrell DA, Ward SG. PI 3-K and T-cell activation: limitations of T-leukemic cell lines as signaling models. Trends Immunol. 2001; 22:490–496. [PubMed: 11525939]
19. Bain J, et al. The selectivity of protein kinase inhibitors: a further update. Biochem J. 2007; 408:297–315. [PubMed: 17850214]
20. Garcia-Echeverria C, Sellers WR. Drug discovery approaches targeting the PI3K/Akt pathway in cancer. Oncogene. 2008; 27:5511–5526. [PubMed: 18794885]
21. Cheng JQ, Lindsley CW, Cheng GZ, Yang H, Nicosia SV. The Akt/PKB pathway: molecular target for cancer drug discovery. Oncogene. 2005; 24:7482–7492. [PubMed: 16288295]
22. Peifer C, Alessi DR. Small-molecule inhibitors of PDK1. Chem Med Chem. 2008; 3:1810–1838. [PubMed: 18972468]
23. Xu Z, et al. Development of high throughput HTRF and Alphascreen assays for identification of potent inhibitors of PDK1. J Biomol Screen. 2009 in the press.
24. Sun D, et al. Efficient identification of novel leads by dynamic focused screening: PDK1 case study. Comb Chem High Throughput Screen. 2009 in the press.
25. Bilodeau MT, et al. Allosteric inhibitors of Akt1 and Akt2: a naphthyridinone with efficacy in an A2780 tumor xenograft model. Bioorg Med Chem Lett. 2008; 18:3178–3182. [PubMed: 18479914]
26. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science. 2002; 298:1912–1934. [PubMed: 12471243]
27. Nurse P. Universal control mechanism regulating onset of M-phase. Nature. 1990; 344:503–508. [PubMed: 2138713]
28. Pan ZQ, Amin A, Hurwitz J. Characterization of the *in vitro* reconstituted cyclin A or B1-dependent cdk2 and cdc2 kinase activities. J Biol Chem. 1993; 268:20443–20451. [PubMed: 8397207]
29. Obenauer JC, Cantley LC, Yaffe MB. Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. Nucleic Acids Res. 2003; 31:3635–3641. [PubMed: 12824383]
30. Daile P, Carnegie PR, Young JD. Synthetic substrate for cyclic AMP-dependent protein kinase. Nature. 1975; 257:416–418. [PubMed: 170533]
31. Daile P, Carnegie PR. Peptides from myelin basic protein as substrates for adenosine 3′, 5′-cyclic monophosphate-dependent protein kinases. Biochem Biophys Res Commun. 1974; 61:852–858. [PubMed: 4141623]
32. Kemp BE, Pearson RB. Design and use of peptide substrates for protein kinases. Methods Enzymol. 1991; 200:121–134. [PubMed: 1835511]
33. Goldstein DM, Gray NS, Zarrinkar PP. High-throughput kinase profiling as a platform for drug discovery. Nat Rev Drug Discov. 2008; 7:391–397. [PubMed: 18404149]
34. Schwartz D, Gygi SP. An iterative statistical approach to the identification of protein phosphorylation motifs from large-scale data sets. Nat Biotechnol. 2005; 23:1391–1398. [PubMed: 16273072]
35. Manning BD, Cantley LC. Hitting the target: emerging technologies in the search for kinase substrates. Sci STKE. 2002:PE49. [PubMed: 12475999]
36. Parang K, Kohn JA, Saldanha SA, Cole PA. Development of photo-crosslinking reagents for protein kinase-substrate interactions. FEBS Lett. 2002; 520:156–160. [PubMed: 12044889]
37. Shen K, Cole PA. Conversion of a tyrosine kinase protein substrate to a high affinity ligand by ATP linkage. J Am Chem Soc. 2003; 125:16172–16173. [PubMed: 14692742]
38. Linding R, et al. Systematic discovery of in vivo phosphorylation networks. Cell. 2007; 129:1415–1426. [PubMed: 17570479]
39. Johnson SA, Hunter T. Kinomics: methods for deciphering the kinome. Nat Methods. 2005; 2:17–25. [PubMed: 15789031]
40. Maly DJ, Allen JA, Shokat KM. A mechanism-based cross-linker for the identification of kinase-substrate pairs. J Am Chem Soc. 2004; 126:9160–9161. [PubMed: 15281787]

41. Statsuk AV, et al. Tuning a three-component reaction for trapping kinase substrate complexes. J Am Chem Soc. 2008; 130:17568–17574. [PubMed: 19053485]
42. Domon B, Aebersold R. Mass spectrometry and protein analysis. Science. 2006; 312:212–217. [PubMed: 16614208]
43. Andersen JS, et al. Proteomic characterization of the human centrosome by protein correlation profiling. Nature. 2003; 426:570–574. [PubMed: 14654843]
44. Andersen JS, et al. Nucleolar proteome dynamics. Nature. 2005; 433:77–83. [PubMed: 15635413]
45. Foster LJ, et al. A mammalian organelle map by protein correlation profiling. Cell. 2006; 125:187–199. [PubMed: 16615899]
46. Rikova K, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell. 2007; 131:1190–1203. [PubMed: 18083107]
47. Wilson-Grady JT, Villen J, Gygi SP. Phosphoproteome analysis of fission yeast. J Proteome Res. 2008; 7:1088–1097. [PubMed: 18257517]
48. Zhai B, Villen J, Beausoleil SA, Mintseris J, Gygi SP. Phosphoproteome analysis of *Drosophila melanogaster* embryos. J Proteome Res. 2008; 7:1675–1682. [PubMed: 18327897]
49. Dephoure N, et al. A quantitative atlas of mitotic phosphorylation. Proc Natl Acad Sci USA. 2008; 105:10762–10767. [PubMed: 18669648]
50. Olsen JV, et al. Global, *in vivo*, and site-specific phosphorylation dynamics in signaling networks. Cell. 2006; 127:635–648. [PubMed: 17081983]

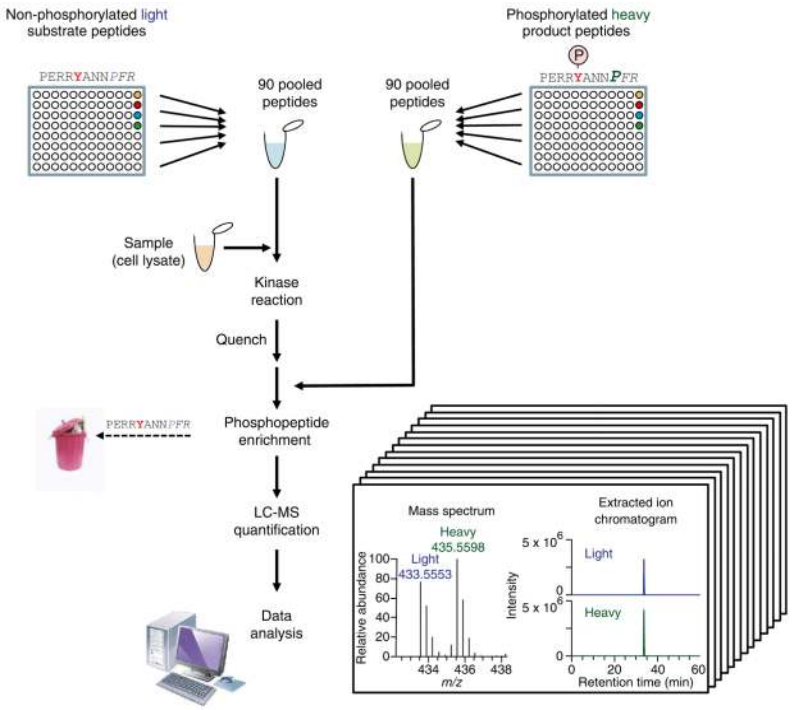

Figure 1.

Workflow for a single-reaction, 90-substrate *in vitro* kinase assay. Synthetic substrate peptides are pooled and incubated with cell lysate. After kinase reactions are quenched, stable isotope–labeled phosphopeptides (internal standards; heavy label on italicized proline) of identical sequence to substrate peptides are added at a known concentration. Phosphorylated substrate peptides and internal standard phosphopeptides are enriched using immobilized metal-ion affinity chromatography and analyzed by LC-MS techniques. Pairs of light (product) and heavy (internal standard) peptides co-elute perfectly, but because they differ in mass by 6 Da, can be quantified by direct ratio of light-to-heavy areas under the curve from high-resolution data. Each assay produces 90 activity measurements in core signaling pathways.

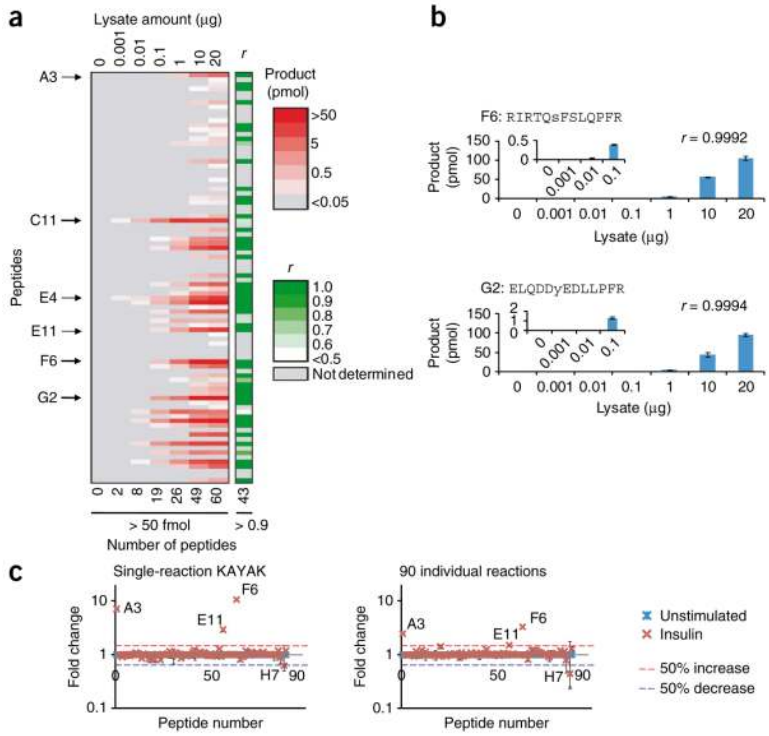

Figure 2.

Sensitivity and reproducibility of the single-reaction KAYAK assay. (**a**) Sensitivity and linearity of the 90-peptide KAYAK approach. Seven different amounts of lysate from HEK-293 cells treated with insulin were used. Product amounts are shown as a heat map of white to red. Products of <50 fmol were empirically considered not observed (gray). The Pearson product-moment correlation coefficients for lysate-to-product amounts for each peptide are shown using green intensity scaling. (**b**) Examples of peptides from **a**, including a serine-phosphorylated peptide (F6) and a tyrosine-phosphorylated peptide (G2). The data are shown as means of duplicates with error bars to the minimum and maximum values. (**c**) Comparison between single-reaction (competing peptides) and 90 individual kinase assays (no competition). The fold change for each peptide's activity measurement for HEK-293 cells with and without insulin treatment is shown. Each reaction involved 20 µg of lysate. Product amounts were normalized to untreated cell lysate and are displayed as means ± s.d. (n = 3).

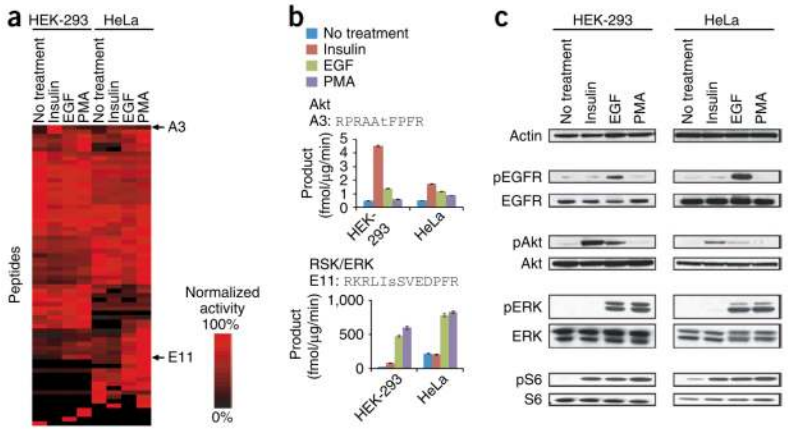

Figure 3.

Induced core pathway phosphorylation changes in human cell lines are faithfully reported by profiling using the single-reaction KAYAK assay. (**a**) Heat map of triplicate KAYAK activity experiments. Kinase activities using lysates (20 µg) from HEK-293 cells and HeLa cells untreated or treated with insulin, EGF or PMA were measured using 90 peptides. The phosphorylation rates for the 68 observed peptides were normalized by reference to the most phosphorylated sample and analyzed by Pearson coefficient hierarchical clustering to group similar responders together. Each row represents the phosphorylation rate of a different peptide normalized to the highest value in the row. The fold change representations from 'No treatment' are shown in Supplementary Figure 6. (**b**) Examples of peptides in **a**. The data are shown as averages ± s.d. (n = 3). Candidate kinases are listed based on phosphorylation using purified kinases (Supplementary Fig. 1). (**c**) Western blot analysis of the lysates using antibodies as indicated. Full length blots are presented in Supplementary Figure 21.

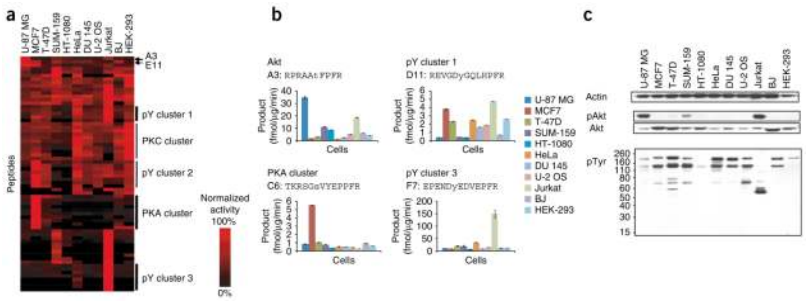

Figure 4.

Profiling of 11 human cell lines using the single-reaction KAYAK assay demonstrates heterogeneity in basal kinase activities and activation state of core pathways. (**a**) Heat map of kinase activities. The 11 cell lines included U-87 MG (glioblastoma), MCF7 (breast), T-47D (breast), SUM-159 (breast), HT-1080 (fibrosarcoma), HeLa (cervical), DU 145 (prostate), U-2 OS (osteosarcoma), Jurkat (T lymphocyte), BJ (foreskin fibroblast) and HEK-293 (embryonic kidney). Each was cultured under ATCC recommended or equivalent conditions and lysed. Lysates (20 µg) were subjected to KAYAK profiling. Using 68 peptides with observable phosphorylation, activities were normalized to the highest value in each row, followed by hierarchical cluster analysis, which groups peptides with similar responses together. (**b**) Examples of several peptides from **a**. The data are shown as the mean from duplicate analyses with minimum and maximum values as error bars. (**c**) Western blot analysis of the lysates using antibodies as indicated. Full-length blots are presented in Supplementary Figure 21.

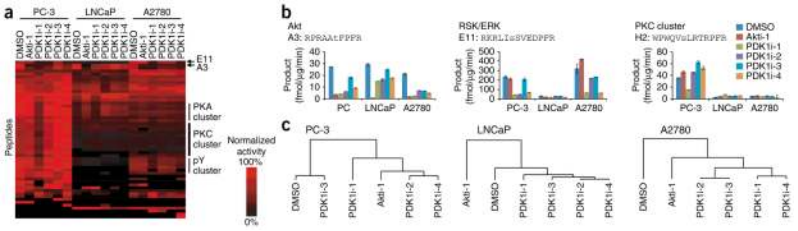

Figure 5.

Cancer cell lines with elevated Akt activity differ markedly in their response to inhibitors of Akt and PDK1. (**a**) Heat map of normalized kinase activities. Three commonly-used cell lines (PC-3, LNCaP and A2780) with a constitutively active PI3K/Akt pathway were treated with DMSO, an allosteric Akt inhibitor (Akti-1) or one of four PDK1 inhibitors (PDK1i-1–4) (Supplementary Fig. 12 for compound information). Kinase activities were profiled using 10 μg of each cell lysate and 90 KAYAK peptides. The phosphorylation rates for 54 observed peptides were normalized to the highest value for that peptide followed by hierarchical cluster analysis. (**b**) Examples of select peptides from panel **a**. The data are shown as the mean of duplicates with error bars to minimum and maximum values. (**c**) Dendrograms of activity profiles in response to inhibitor treatment for the three cell lines.

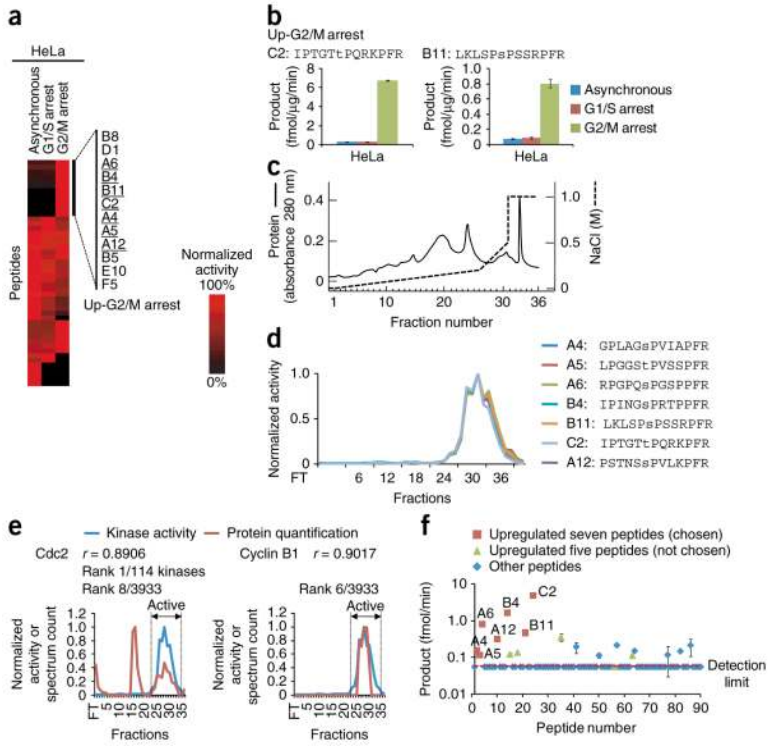

Figure 6.

Identification of Cdc2/Cyclin B1 complex as an activated kinase in mitosis. (**a**) Heat map of kinase activities from cell cycle lysates. HeLa cells were cultured under standard conditions (asynchronous), or synchronized in either G1/S or G2/M phase of the cell cycle. Kinase activities from lysates (20 µg) were analyzed by KAYAK profiling. Phosphorylation rates were normalized and clustered as in Figure 3. (**b**) Examples of peptides in **a**. Seven peptides (underlined in **a**) showing this pattern were chosen for correlation profiling to identify the mitotic kinase. (**c**) UV chromatogram of protein elution into 36 fractions from the anion exchange column using G2/M phase cell lysate. (**d**) Kinase activity profile (normalized to the highest value) using seven upregulated peptides and the fractions in panel **c**. (**e**) Correlation profiles of kinase activity and protein quantification. 3,933 proteins (114 kinases) were identified by 'shotgun' LC-MS/MS analysis of flow-through (FT) and 36 fractions. Protein amount was estimated based on peptide identifications (Online Methods) and normalized to the highest value. Correlation profiling ranked Cdc2 as the most likely kinase (1/114) and eighth best ranked protein overall (8/3933). In addition, the amount of Cyclin B1 was highly correlated. *r*-values represent Pearson product-moment correlation coefficients between peak kinase activity and protein abundance in active fractions. (**f**) KAYAK profiling of 90 peptides using purified Cdc2/Cyclin B1. The product amounts for the seven peptides in panel **d** are shown as red squares.