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A Multiplexed NMR-Reporter Approach to Measure Cellular Kinase and Phosphatase Activities in Real-Time

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Supporting Information

ABSTRACT: Cell signaling is governed by dynamic changes in kinase and phosphatase activities, which are difficult to assess with discontinuous readout methods. Here, we introduce an NMR-based reporter approach to directly identify active kinases and phosphatases in complex physiological environments such as cell lysates and to measure their individual activities in a semi-continuous fashion. Multiplexed NMR profiling of reporter phosphorylation states provides unique advantages for kinase inhibitor studies and reveals reversible modulations of cellular enzyme activities under different metabolic conditions.

Kinases and phosphatases determine the reversible phosphorylation states of a vast number of proteins and thereby regulate many aspects of cell signaling.[1] To measure such dynamic enzymatic activities, analytical methods ought to be able to continuously monitor substrate phosphorylation states, which is incompatible with most antibody, mass spectrometry, or fluorescence-based kinase and phosphatase activity assays.[2−5] Here, we present an alternative nondisruptive NMR approach to monitor multiple kinase activities in parallel, over extended periods of time, and in a semicontinuous manner. Similar to other reporter assays,[6] we make use of peptide-based kinase substrates, whose individual phosphorylation states, and time-dependent changes thereof, convey balanced kinase and phosphatase activities. Differently though in our approach, we employ time-resolved NMR spectroscopy to monitor individual reporter phosphorylation states directly in cell lysates, without the need for sample purification or processing.[7] Incorporation of NMR-active isotopes at the respective reporter phosphorylation sites provides the necessary "visualization filter" to simultaneously observe multiple reporters in nonisotope-labeled cellular environments.

To generate kinase activity reporters (KARs) for NMR-based profiling routines, we initially engineered validated kinase consensus sequences as N-terminal extensions to the globular protein G B1 domain (GB1, 6 kDa) (Figure 1a).[8] We recombinantly expressed and purified these substrates as uniformly ^{15}N isotope-labeled GB1-reporters and confirmed

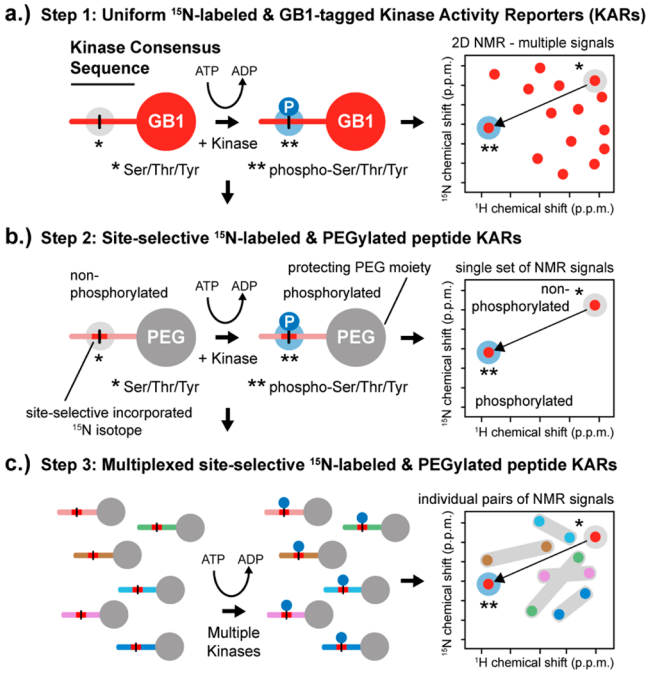

Figure 1. Kinase activity reporter (KAR) design and preparation. (a) Uniform ^{15}N isotope-labeled and GB1-tagged kinase consensus sequences. Phosphorylation leads to characteristic chemical shift changes of modified reporter residues. (b) Site-selective isotope-incorporation and solid-phase peptide synthesis with C-terminal polyethylene glycol (PEG) moieties as protecting groups. (c) KAR chemical shift dispersions are sufficiently large to monitor multiple reporters in parallel.

phosphorylation in reconstituted kinase reactions and in cell lysates of known enzymatic activities, such as *Xenopus laevis* cytostatic factor-arrested (CSF) egg extracts.[9] As shown previously,[8] phosphorylation led to characteristic chemical shift changes of modified reporter residues (Figure S1); however, this information was "congested" with GB1 signals, which rendered simultaneous monitoring of multiple reporters

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impossible. Therefore, we generated peptide versions of KAR sequences by solid-phase synthesis in a next step, omitting the GB1 domain and incorporating ^{15}N isotope-labeled amino acids only at the respective kinase phosphorylation sites. By doing so, we reduced the spectral complexity of each reporter to a single NMR signal (Figure 1b). Given the unique kinase-consensus-sequence-context of each reporter, we expected NMR signals of different KARs to be sufficiently well dispersed to monitor multiple reporters in parallel (i.e., multiplexing) (Figure 1c). KAR sequences were chosen based on extensive cross-reactivity tests and iterative optimization routines to enhance kinase specificity (Figure S2 and SI Note 1). For tyrosine kinase reporters, we incorporated ^{15}N isotope labels at positions N- or C-terminal to the phosphorylatable amino acid because phosphorylation-induced chemical shift changes were largest for residues adjacent to the modified tyrosines (Figure S3).[10] In cell lysates, peptide KARs were initially prone to rapid degradation (Figure S4a), which we overcame by synthesizing reporters with C-terminal polyethylene glycol (PEG, ~3 kDa) moieties as protecting groups (Figure 1b). PEGylated KARs proved stable over extended periods of time (>12 h) and displayed phosphorylation efficiencies comparable to GB1-tagged reporters (Figure S4b). We further established that time-resolved NMR readouts of KAR phosphorylation states robustly delineated kinase activities as low as 10 enzymatic unit (U) equivalents and activity differences of 20 U (Figure S4c).

To illustrate the power of multiplexed KAR assays, we initially measured phosphorylation of eight reporters in separate aliquots of CSF extracts (Figure 2a). In line with previous results, we found high Cyclin-dependent protein kinase 1 (p34/Cyclin B, or Cdk1),[11] mitogen-activated protein kinase (p42/MAPK),[12] p90 ribosomal S6 kinase 2 (Rsk2),[13] Casein kinases 1 and 2 (CK1, CK2),[14,15] checkpoint kinase 1 (Chk1)[16] and Polo-like kinase 1 (Plk1)[17] activities, which led to complete reporter phosphorylation within ~60 min of extract incubation. Modification of the 3-phosphoinositide-dependent protein kinase 1 (PDK1)[18] reporter was least efficient and leveled off before reaching 100%. Next, we measured the same set of KARs in one aliquot of CSF extract and determined virtually identical modification trajectories (Figure 2b). These results demonstrate that multiplexed KAR monitoring by time-resolved NMR spectroscopy offers robust means to derive qualitative and quantitative information about active kinases in single aliquots of cell lysates, without adverse effects on profiling accuracy or reproducibility.

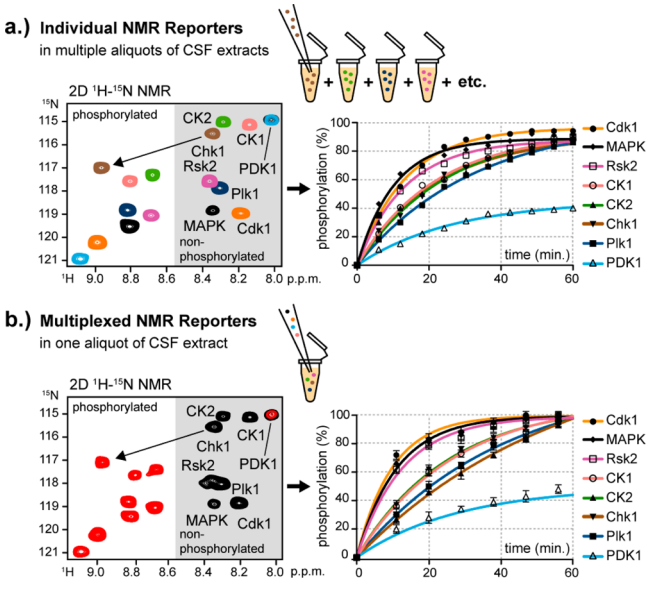

Figure 2. Multiplexed KAR profiling in cell lysates. (a) Time-resolved NMR readouts of KAR phosphorylation in separate aliquots of CSF extracts. Superposition of corresponding 2D NMR spectra illustrates the degree of spectral overlap of unmodified and phosphorylated KARs. Plotting of phospho-KAR signals against time produces individual modification curves. (b) Multiplexed reporters in one aliquot of CSF extract. Measurement points with error bars represent mean values of duplicate experiments. Error bars are SD.

The ability to directly measure multiple kinase activities in parallel has obvious advantages for determining the potencies and specificities of kinase inhibitors, the second largest class of drug targets. To illustrate this point, we analyzed commercial Cdk1 inhibitors in multiplexed CSF-extract reactions (Figure 3). Inhibitor A (see SI Materials and Methods for details) led to

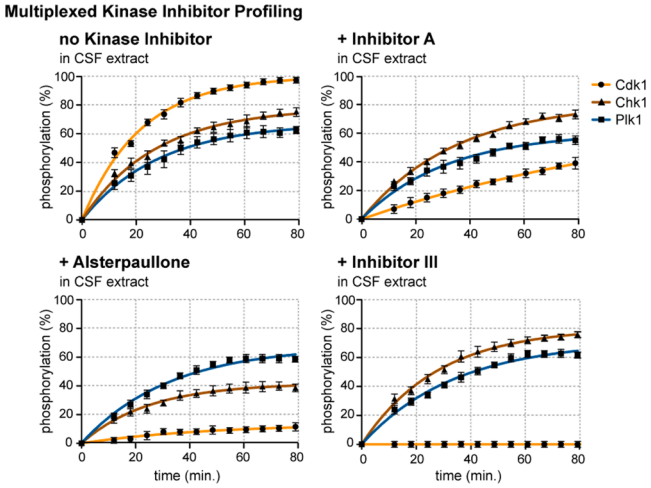

Figure 3. KAR-based kinase inhibitor profiling. Multiplexed KAR profiling in the presence of different Cdk1 inhibitors reveals compound specificity and cross-reactivity against endogenous Plk1 and Chk1. Measurement points with error bars represent mean values of duplicate experiments. Error bars are SD.

partial inhibition of Cdk1 and slight cross-inhibition of Plk1, whereas Alsterpaullone reduced Cdk1, but also Chk1 activity. Inhibitor III abolished Cdk1 activity without affecting Chk1 and Plk1, thus highlighting the suitability of multiplexed KAR approaches to quantify kinase inhibitor efficacies and off-target effects in cell lysates.

NMR monitoring of KAR phosphorylation states is fully reversible and also reveals dephosphorylation by recombinant and cellular phosphatases (PPase). Accordingly, prephosphorylated KARs can be used to measure phosphatase activities *in vitro* and in cell lysates. We found that phosphorylated KARs were excellent substrates for PP2A and lambda phosphatase (λ-PPase) and that they were efficiently dephosphorylated by endogenous enzymes in CSF extracts (Figure 4a). This indicated that KAR phosphorylation levels in cell lysates reflect the combined influence of both classes of enzymes and may therefore plateau below 100% when kinases and phosphatases jointly act on the same reporter. To exemplify this notion, we reconstituted phosphorylation reactions, in which one defined kinase activity acted against increasing amounts of phosphatase. Mathematical modeling of the behavior of such a system

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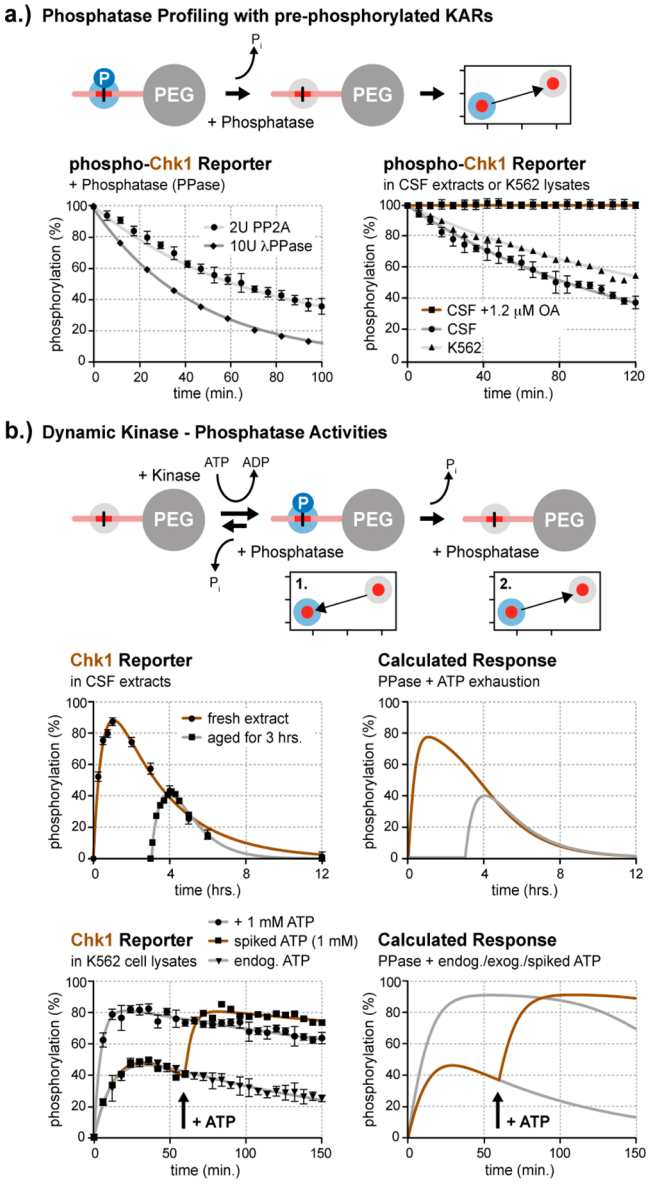

Figure 4. KAR profiling of kinase and phosphatase activities. (a) Prephosphorylated KARs to measure phosphatase activities in reconstituted enzyme reactions and cell lysates. Phosphatase inhibition with okadaic acid (OA) preserves phosphorylation of the Chk1 reporter in CSF extracts. (b) Progressive ATP depletion in CSF extracts leads to declining kinase activities without affecting phosphatases, which results in dynamic KAR phosphorylation states in response to changing ATP levels. Timed ATP spiking experiments in K562 cell lysates confirm this dependency. Calculated response behaviors agree with experimentally determined modification profiles. Measurement points with error bars represent mean values of duplicate experiments. Error bars are SD.

predicted incremental reductions in maximum phosphorylation levels, which we experimentally confirmed by reacting the Chk1 reporter with 500 U of kinase in the presence of different amounts of λ-PPase (Figure S5 and SI Note 2). These data show that NMR readouts of KAR modification states accurately reproduce the combined effect of balanced kinase and phosphatase activities.

ATP levels in CSF extracts and mammalian cell lysates decrease rapidly (Figure S6a,b). In turn, progressive ATP depletion results in the inability of cellular kinases to continuously phosphorylate their reporters. In the presence of phosphatases, this leads to KAR phosphorylation being followed by dephosphorylation because initial kinase activities are eventually superseded by phosphatase contributions (Figure 4b). To determine whether such lysate conditions can be used to mimic dynamic signaling behaviors, we incubated KARs known to undergo dephosphorylation by cellular phosphatases with CSF extracts over extended periods of time, or upon preaging of cell extracts for 3 h. Time-resolved NMR experiments confirmed that initial KAR phosphorylation was followed by dephosphorylation, in line with modeled response behaviors of declining kinase activities acting against constant phosphatase activities (Figures 4b and S6c and SI Note 2).

ATP-spiking experiments in mammalian K562 cell lysates further substantiated the influence of ATP on KAR phosphorylation levels in physiological mixtures containing both kinases and phosphatases (Figure 4b). Indeed, our experimental modification profiles were in good agreement with the calculated response curves that we obtained when taking the measured rates of ATP hydrolysis into account (Figure S6b and SI Note 2). Thus, NMR monitoring of KAR phosphorylation states is well suited to convey dynamic changes of endogenous kinase and phosphatase activities in complex physiological mixtures.

In summary, we introduced a peptide-based NMR-reporter approach to measure cellular kinase and phosphatase activities in a time-resolved manner. By employing solid-phase peptide synthesis, site-selective ^{15}N isotope incorporation, and PEG-stabilization, kinase and phosphatase activity reporters can be generated for a variety of applications, including multiplexed kinase inhibitor profiling as one example. Tyrosine kinase reporters are less specific than serine- and threonine-KARs, and their use may be restricted to identifying active kinase families, rather than to distinguishing between individual kinase family members. For multiplexed KAR measurements, the maximum number of reporters that can be used in one reaction setup is given by the spectral overlap of their NMR signals. Because resonance positions of "unmodified" and "phosphorylated" reporter signals are known from reference experiments with recombinant kinases, specific sets of KARs can be combined to avoid signal overlap. When kinase activities in cell lysates are studied, different batches of mixed KARs may be used on separate sample aliquots to achieve comprehensive coverage. In contrast to assays that require sample purification and enrichment of phosphorylated peptide reporters,[3] the absolute amounts of unmodified and modified KAR molecules are detected side-by-side in each NMR spectrum of the reaction time-series, with individual signal intensities corresponding to the concentrations of unmodified and phosphorylated reporters. This renders KAR profiling of cellular kinase activities highly accurate, as we have also shown by direct comparison with semiquantitative Western blotting.[19] Given the excellent agreement of measured KAR modification profiles with calculated response behaviors of individual kinases, dynamic signaling activities can be analyzed in a quantitative manner and with unprecedented temporal resolution. Underscoring the broad applicability of the method, the design and use of KARs is straightforward, and targeted peptide libraries against different sets of kinases can be generated with ease.

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■ ASSOCIATED CONTENT

Supporting Information

Supplementary Figures S1−S6, Materials and Methods, explanatory notes for KAR design and cross-reactivity testing (SI Note 1), and mathematical modeling (SI Note 2). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b02987.

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Notes

The authors declare no competing financial interest.

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