A multiplexed homogeneous fluorescence-based assay for protein kinase activity in cell lysates

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New methods to quantify protein kinase activities directly from complex cellular mixtures are critical for understanding biological regulatory pathways. Herein, a fluorescence-based chemosensor strategy for the direct measurement of kinase activities in crude mammalian cell lysates is described. We first designed a new fluorescent peptide reporter substrate for each target kinase. These kinase chemosensors were readily phosphorylated by recombinant target enzyme and underwent a several-fold fluorescence increase upon phosphorylation. Then, using unfractionated cell lysates, a homogeneous kinase assay was developed that was reproducible, linear and highly preferential for monitoring changes in cellular activity of the target kinase. The general protocol was developed for the kinase Akt and then easily extended to measure protein kinase A (PKA) and mitogen-activated protein kinase–associated protein kinase 2 (MK2) activities. This assay platform is immediately useful for studying protein kinase signaling in crude cellular extracts.

Protein phosphorylation is a vital mechanism of intracellular regulation[1]. Protein kinases initiate and relay phosphorylation signals along intracellular pathways. As the catalytic activities of these enzymes are the clearest measure of signaling information 'flow'[2], there is tremendous interest in quantifying the dynamics of kinase activities in complex cellular media.

Many kinases are themselves regulated by phosphorylation, and measurements using phosphospecific antibodies to the kinase are widely used to approximate catalytic activity. But this approach neglects other post-translational modifications and protein-protein associations that might influence kinase activity *in vivo*[3–5].

The most common methods for measuring cellular kinase activities involve isolation of the target kinase from cell lysates[4,5]. Then, *in vitro* kinase assays are performed with protein or peptide substrates and [γ-^{32}P]ATP, and autoradiography or liquid scintillation counting is used to quantify the phosphorylated product. Limitations of this format are that the kinase reaction is a 'quenched-point' assay, and processing steps needed to isolate the kinase and measure activity are time-consuming and inherently have low throughput. Moreover, sub-physiological ATP concentrations are required (10–50 μM) to avoid overdiluting the [γ-^{32}P]ATP. Such 'ATP-starved' conditions diminish the absolute activity of most kinases, which reduces the resolution and sensitivity of radioactivity-based kinase assays.

To more directly and rapidly analyze crude cell lysates, a continuous, fluorescence-based, homogeneous kinase activity assay is very desirable. Despite numerous reports of kinase sensors in the literature, only a fraction are compatible with unfractionated cell lysates or within cells[6–18]. Moreover, all of these assays either (i) show small fluorescence changes, (ii) involve nonversatile design strategies, or (iii) require technically challenging microfluidic setups. Thus, despite the recognized importance of protein kinases, there is not a straightforward way to quantify kinase activities from cells.

Previously, we described a versatile and sensitive fluorescence-based chemosensor strategy for monitoring recombinant kinase activity *in vitro*[19]. The chemosensor comprises a small sensing module appended to an optimized peptide substrate for the target kinase. The fluorescence signal is generated when the non-natural Sox amino acid[20] undergoes chelation-enhanced fluorescence in the presence of divalent magnesium (**Fig. 1a**). The Mg^{2+}-binding affinity of the product phosphopeptide is much greater than that of the substrate peptide, which results in a large fluorescence increase upon phosphorylation[19].

Here we report that the sensitivity and selectivity of Sox-based chemosensors are sufficient to measure kinase activities directly from unfractionated cell lysates. Using similar design principles, we engineered new Sox-based fluorescent substrates for three important protein kinases: Akt[21,22], MK2 (refs. 23 and 24) and PKA[25,26]. These chemosensors are excellent reporters of both recombinant enzyme activity *in vitro* and endogenous activity *ex vivo*. We established optimized assay conditions for preferential, quantitative detection of cellular Akt, MK2 and PKA activation. The homogeneous assay format is high-throughput, remarkably straightforward and reproducible; notably, it is also compatible with physiological concentrations of ATP (1 mM). This work establishes an important tool for studying the roles of protein kinases in cellular regulation.

RESULTS

Design and validation of fluorescent kinase chemosensors

The kinase chemosensor peptides (Akt-S1, MK2-S1 and PKA-S3; **Table 1**) were developed from optimized peptide substrates for

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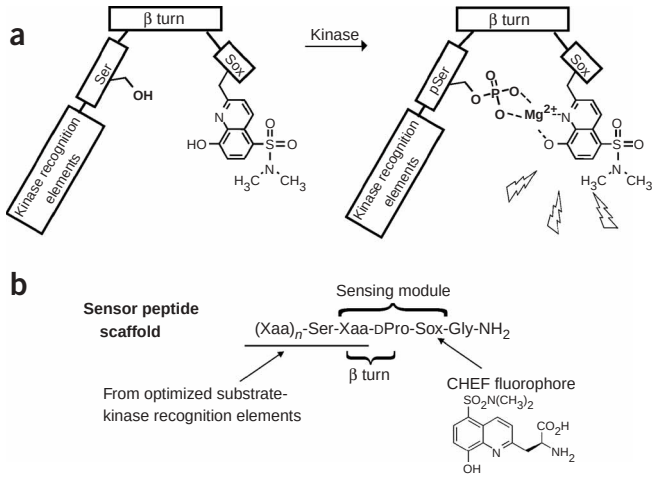

Figure 1 | Design of fluorescent chemosensors of Akt, MK2 and PKA activity. (**a**) The chemosensor peptide contains three important design modules for fluorescence sensing: critical kinase specificity determinants (kinase recognition elements), the chelation-enhanced fluorophore Sox[20] and a β-turn to preorganize Mg^{2+}-binding between Sox and the incipient phosphate. Phosphorylation increases the affinity of the peptide for Mg^{2+} and the fluorescent signal is generated by chelation of Mg^{2+}. (**b**) The critical kinase specificity determinants in the sensor peptide sequence are derived from an optimized peptide substrate, including one residue in the sensing module as part of the β-turn sequence.

Akt[27], MK2 (ref. 28) and PKA[29] (**Fig. 1b**). For each chemosensor, the N-terminal portion of the optimized substrate was preserved to retain the consensus motif for substrate recognition. The sensing module was appended C-terminal to the target serine. Notably, the β-turn element in the sensing module tolerates one additional amino acid recognition element for improved specificity and reactivity.

To evaluate the fluorescence increase and kinetic parameters of each substrate, both substrate peptides and phosphoserine (pS)-containing product peptides (Akt-P1, MK2-P1 and PKA-P3) were synthesized. When excited at 360 nm under the final assay conditions, the phosphopeptides were five- to tenfold more fluorescent (λ_{em} = 485 nm) than the corresponding unphosphorylated peptides at identical concentrations (**Table 1**). The absolute fluorescence increases depend on the instrumentation (**Supplementary Methods** online), concentration of Mg^{2+}, presence of other chelators and ionic strength. Compared with that for other sensors, this fluorescence increase is over tenfold greater than most[6–17] and over threefold larger than that for the most sensitive probe used to date[18]. By measuring fluorescence[19], it was determined that these new chemosensors retained the high activity of the original optimized peptides[27–29] (**Table 1**).

Development of an Akt-S1 kinase activity assay

The sensitivity and efficacy of these kinase chemosensors prompted an investigation of how each would perform in more complex environments. Whereas direct *in vivo* applications are a common next step for most kinase activity sensors[6–17], there are several advantages to testing sensor properties in cell extracts. For instance, (i) sensitivity and specificity can be improved by adding inhibitors of phosphatases and off-target kinases, (ii) quantification of fluorescence changes is more straightforward, and (iii) many distinct lysate-based assays can be performed using different aliquots of the same sample.

We first developed an Akt-S1 activity assay because this chemosensor had the largest fluorescence increase after phosphorylation. An initial concern for a homogeneous assay was compatibility between the lysis buffer, containing crude cellular proteins, nonionic detergents and various inhibitors, and a typical kinase assay buffer[2], containing kinase and phosphatase inhibitors, ATP and substrate (see **Supplementary Methods** for details). Typically, 40–100 μg of cell lysate in 7.5% of the total reaction volume was used in the Akt-S1 assay. Radioactive Akt assays[2,3] typically use 500 μg lysate and micromolar concentrations of ATP. Here less lysate is needed because the Akt-S1 assay is compatible with 1 mM ATP. We found that assay sensitivity improved by an order of magnitude when the ATP concentration was increased from 10 μM to 1 mM (**Supplementary Fig. 1** online).

Another challenge for a crude lysate assay was the presence of competing cellular processes, such as off-target kinases and phosphatases. To improve specificity for Akt, a cocktail of phosphatase and kinase inhibitors was included in the assay buffer (see Methods). We confirmed that none of these kinase inhibitors affected recombinant Akt1 activity, but they completely inhibited their target enzymes (**Supplementary Fig. 2** online). For the kinase assay, it was critical to add the small-molecule bisindolylmaleimide protein kinase C (PKC) inhibitor GF109203X, which targets all PKC isozymes as well as p70S6 kinase and MAPKAP-K1 (ref. 30; **Supplementary Methods**).

The Akt-S1 kinase activity assay was optimized in a fluorescence microcuvette and then adapted to a 96-well-glass-plate format (see Methods and **Supplementary Methods**). To obtain Akt activity measurements, cell lysates were added to the complete assay mixture, and fluorescence readings were taken each minute for 60 min. The change in fluorescence was linear during this time (**Fig. 2a**), and the slope was used as the measure of activity because it is the best aggregate metric for the increase in fluorescence. All subsequent data were collected in this high-throughput format.

Table 1 | Chemosensor sequences, sensitivities and kinetic parameters

Sensor	Sequence	Fold fluorescence increase	K_M (μM)	V_{max} (μmol min^{-1} mg^{-1})
Akt-S1	AcARKRERAY**S**F-DPro-Sox-Gly-NH_2	9.3	3.8 ± 0.1	0.59 ± 0.08
MK2-S1	AcAHLQRQL**S**I-DPro-Sox-Gly-NH_2	8.5	21 ± 1	2.3 ± 0.1
PKA-S3	AcLRRA**S**L-DPro-Sox-Gly-NH_2	5.6	2.9 ± 0.3	6.3 ± 0.3

The underlined portion of each chemosensor sequence indicates the series of amino acids from the optimized peptide substrates Aktide[27], MK2tide[28] and Kemptide[29], and the phosphorylatable serine is in boldface. Fluorescence enhancements and kinetic parameters (K_M and V_{max}, reported as the mean ± s.e.m. for triplicate experiments) were determined as described in Methods.

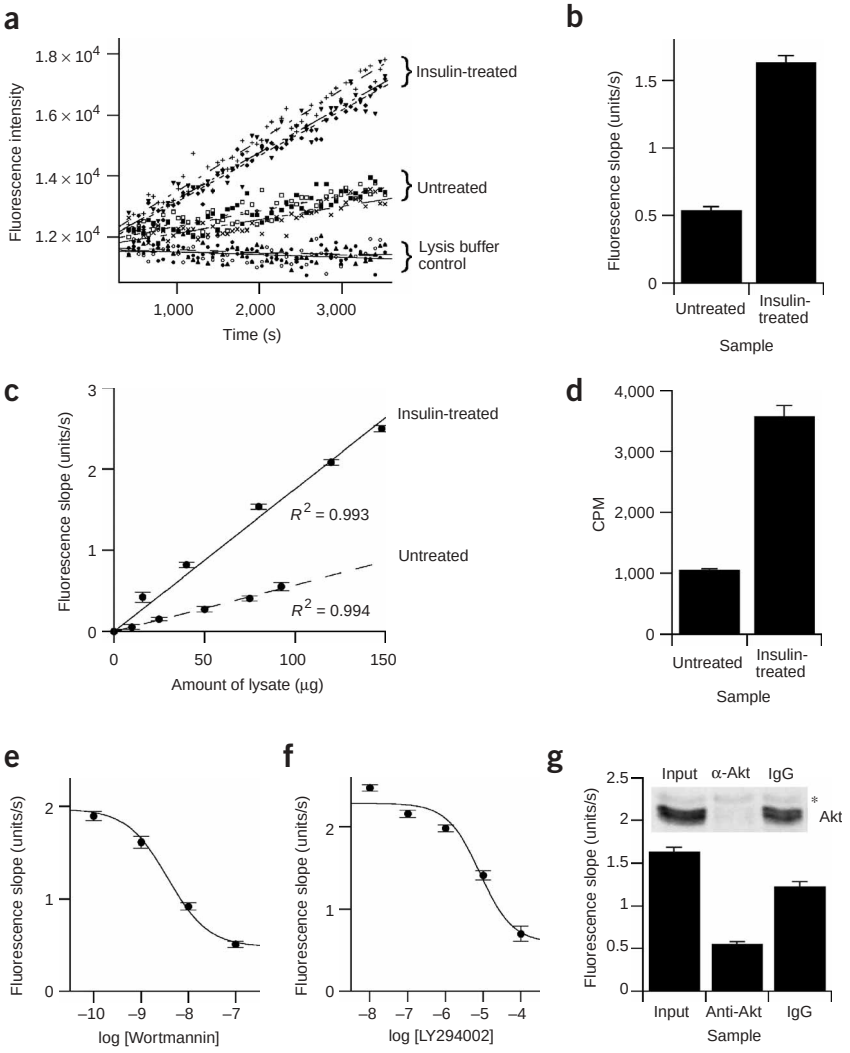

Figure 2 | Akt-S1 kinase activity is quantitatively linear and preferential for Akt. (**a**) Triplicate fluorescence measurements of lysis buffer, untreated HT-29 lysates, and insulin-stimulated HT-29 lysates over 60 min. R^2 values range from 0.72 to 0.97 (**b**) Summary of fluorescence slopes from data in **a**. (**c**) Summary of fluorescence slopes for the insulin-treated and untreated samples that were diluted in lysis buffer to vary the total protein content; the volume of lysis buffer was kept constant for all data points. (**d**) Results of a radioactive assay[2] using the same lysates as in **a**. (**e**,**f**) HT-29 lysates were pretreated with various concentrations of the PI3K inhibitors wortmannin (**e**) and LY294002 (**f**) for 1 h before stimulation with 500 ng/ml insulin. The carrier (DMSO) was kept constant at 0.1% for all stimulation conditions. (**g**) Kinase activity was measured from an insulin-treated HT-29 lysate (input), and after immunodepletion of this lysate with anti-Akt1/2 or naive mouse IgG. Inset: western blot for Akt in the measured samples (asterisk indicates a nonspecific band). Plotted values indicate the mean ± s.e.m. for triplicate measurements.

Validation of the Akt-S1 kinase assay

After generically characterizing lysate activity, we assessed whether the assay format could be used to quantitatively detect changes in activity from cell samples stimulated with known Akt activators. HT-29 cells were treated with insulin for 5 min and lysed by standard techniques. Then, Akt-S1 fluorescence changes were monitored in triplicate with insulin-stimulated or untreated control lysates (**Fig. 2a**). The assay revealed a threefold increase in Akt-S1 kinase activity (**Fig. 2b**). Using recombinant active Akt1, the untreated and insulin-treated lysates were found to correspond to about 8 ng and 23 ng active Akt1 equivalents per 93 µg lysate, respectively. The amount of turnover in the insulin-treated samples after 60 min was 7%.

The Akt-S1 fluorescence slope varied linearly with dilutions of both insulin-treated and untreated lysates over at least a fivefold concentration range of cellular protein (**Fig. 2c**). In addition, the fold difference between insulin-stimulated and unstimulated activities correlated with the activation measured by a quantitative radioactive assay[2] (**Fig. 2d**). Akt-S1 also could report Akt activity in a quenched-point fluorescence assay with immunopurified Akt (**Supplementary Fig. 3** online). These results indicate that the fluorescence-based readings quantitatively report Akt-S1 kinase activity in cell lysates.

Next we tested whether the Akt-S1 kinase assay was specific for measuring Akt activity in crude biological samples by using two small-molecule inhibitors (wortmannin and LY294002) of phosphatidylinositol 3-kinase (PI3K). PI3K initiates recruitment of Akt to the plasma membrane; this recruitment is required and sufficient for Akt activation[31]. Cells were preincubated with the indicated concentration of each PI3K inhibitor for 1 h, then stimulated with insulin for 5 min. Dose-dependent inhibition of Akt-S1 kinase activity was observed for both wortmannin (**Fig. 2e**; 50% inhibition concentration (IC_{50}) = 3.6 nM) and LY294002 (**Fig. 2f**; IC_{50} = 8.2 µM). These values agreed with reported IC_{50} values for the upstream PI3K inhibition by wortmannin (5 nM) and LY294002 (1.4 µM), considering the difference in ATP concentrations[32]. As wortmannin and LY294002 inhibit PI3K by different mechanisms[32], sensitivity to both strongly implicates a PI3K pathway in Akt-S1 phosphorylation.

Because other PI3K-dependent kinases might nonspecifically phosphorylate Akt-S1, Akt was removed *in vitro* by immunodepletion. Insulin-stimulated lysates were depleted with an Akt-specific antibody, and Akt-S1 kinase activity was compared to both the input lysate and a sample that had been depleted with naive mouse IgG. Akt-immunodepleted lysates showed threefold less activity than naive IgG–immunodepleted lysates (**Fig. 2g**), strongly suggesting that the Akt-S1 signal predominantly is due to Akt-mediated phosphorylation. Immunodepletion was confirmed by western blot analysis with the immunodepleting antibody (**Fig. 2g**, inset), as well as with an independent antibody against Akt (data not shown). Slightly reduced activity in the naive immunodepletion sample was due to nonspecific loss of Akt (~23% loss, estimated by densitometry). Also, there was a small but detectable amount of residual Akt in the Akt-depleted lysates (~5% of input). Therefore, we consider the measurements in the

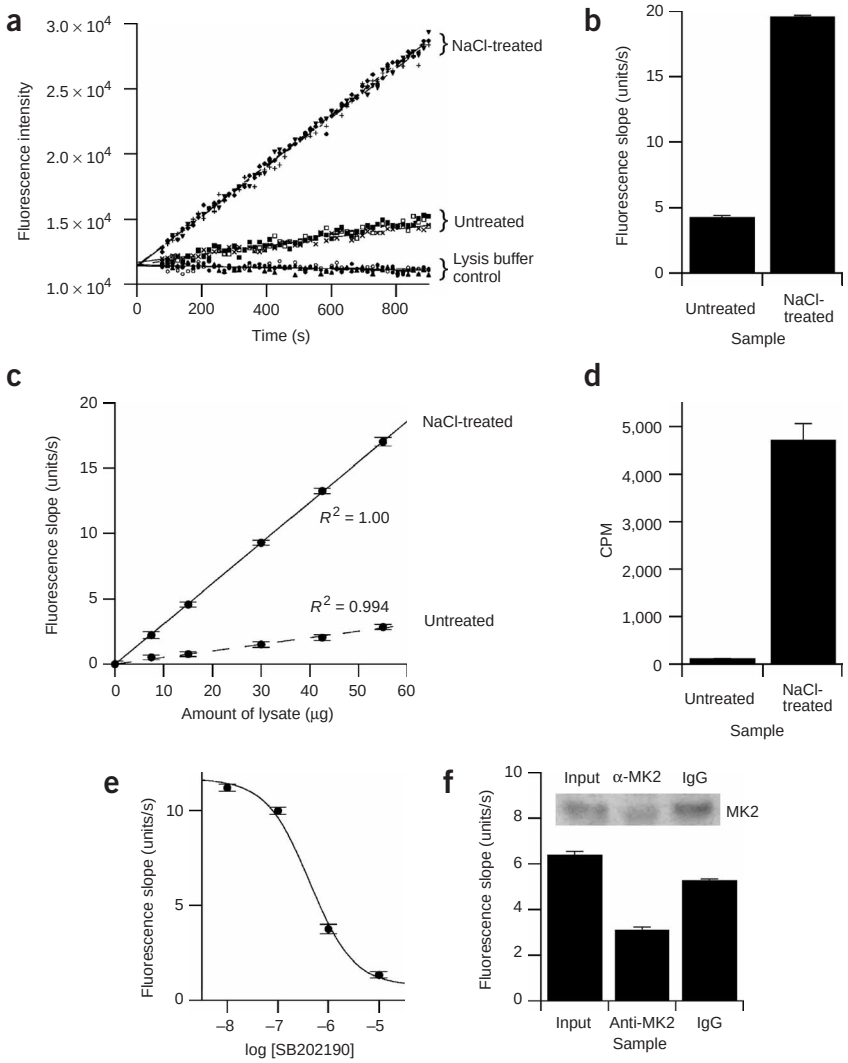

Figure 3 | MK2-S1 kinase activity is quantitatively linear and preferential for MK2. (**a**) Fluorescence measurements of lysis buffer, untreated HT-29 lysates, and NaCl-stimulated HT-29 lysates over 15 min. R^2 values range from 0.93 to 1.0. (**b**) Summary of fluorescence slopes from data in **a**. (**c**) Summary of fluorescence slopes for the NaCl-treated and untreated samples that were diluted in lysis buffer to vary the total protein content; the volume of lysis buffer was kept constant for all data points. (**d**) Results of a radioactive assay[2] using the same lysates as in **a**. (**e**) HT-29 lysates were pretreated with various concentrations of the p38 inhibitor SB202190 for 1 h before stimulation with 250 mM NaCl. The carrier (DMSO) was kept constant at 0.1% for all stimulation conditions. (**f**) Kinase activity was measured from an NaCl-treated HT-29 lysate (input) after immunodepletion of this lysate with anti-MK2 or naive sheep IgG. Inset: western blot for MK2 in the measured samples. Plotted values indicate the mean ± s.e.m. for triplicate measurements.

immunodepleted samples to be a conservative estimate for the selectivity of the assay.

To test the general applicability of the Akt-S1 kinase assay to other mammalian cell types, we measured Akt-S1 activity in Chinese hamster ovary (CHO) cells. The epidermal growth factor (EGF) was used as the Akt agonist (**Supplementary Methods**). Similarly to the insulin-stimulated Akt activity in HT-29 cells (**Fig. 2b**,**d**), we found that EGF-induced Akt activation in CHO cells quantitatively correlated with the activation observed in a radioactive Akt assay[2] (**Supplementary Fig. 4** online). Together these results indicate that the Akt-S1 assay is a quantitative, selective and generalizable format for studying Akt signaling.

Development of MK2-S1 and PKA-S3 kinase activity assays

After successfully developing an Akt-S1 kinase activity assay, we investigated whether the protocol could be applied more broadly to the other target kinases. To design activity assays for PKA and MK2 in HT-29 cell lysates, we used the assay mixture from the Akt assay as a starting point and then refined it for each kinase. We confirmed that the panel of inhibitors for each assay mixture did not affect recombinant MK2 and PKA activities (**Supplementary Fig. 2**).

The MK2-S1 (**Fig. 3**) and PKA-S3 (**Fig. 4**) activity assays were evaluated in HT-29 cell lysates similarly to the Akt-S1 activity assay. The data obtained from both assays were linear for 15 min (**Figs. 3a** and **4a**) and over a fivefold range of lysate concentrations (**Figs. 3c** and **4c**). MK2 activation by NaCl treatment resulted in a 4.6-fold increase in MK2-S1 phosphorylation (**Fig. 3b**). We found that 53 µg of the stimulated lysate contains the equivalent of 51 ng recombinant MK2 with 18% turnover in 15 min. PKA activation by forskolin treatment resulted in a 3.5-fold increase in PKA-S3 phosphorylation (**Fig. 4b**), which corresponds to 12 ng recombinant PKA in 18 µg of HT-29 cell lysate with 9.1% turnover in 15 min.

There was a somewhat weaker correlation between the MK2-S1 assay (**Fig. 3b**) and a radioactive MK2 assay (**Fig. 3d**), so we examined the chemosensor selectivity in detail. MK2-S1 phosphorylation was abrogated in NaCl-treated lysates when cells were pretreated with SB202190, a small-molecule inhibitor of the MK2 kinase p38 (**Fig. 3e**). The apparent IC_{50} value (430 nM) correlated reasonably well with that reported for upstream p38 inhibition (50–100 nM), considering the difference in ATP concentrations[30]. When MK2 was removed *in vitro*, the immunodepleted lysate lost 50% of its activity as compared to that of the input lysate (**Fig. 3f**). In addition, western blot analysis showed that the immunodepleted sample had retained 25% of input MK2, and the naive immunodepleted sample had nonspecifically lost ~15% of input MK2 (**Fig. 3f**, inset). Together, these data suggest that 75% of MK2-S1 activity is due to MK2. The residual 25% of nonspecific activity is likely to explain the higher untreated background activity observed with the MK2-S1 assay relative to the radioactive MK2 assay[2] (**Fig. 3d**). MK2-S1 also reported MK2 activity in a quenched-point fluorescence assay with immunopurified MK2 (**Supplementary Fig. 5** online). Finally, using the MK2-S1 activity assay, we also

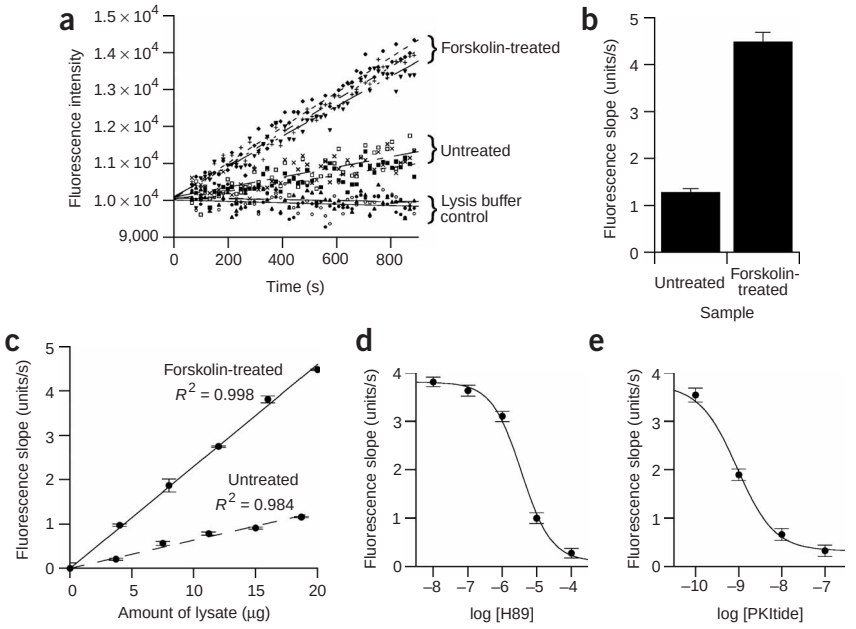

Figure 4 | PKA-S3 kinase activity is quantitatively linear and preferential for PKA. (**a**) Fluorescence measurements of lysis buffer, untreated HT-29 lysates, and forskolin-stimulated HT-29 lysates over 15 min. R^2 values range from 0.60 to 0.97. (**b**) Summary of fluorescence slopes from data in **a**. (**c**) Summary of fluorescence slopes for the forskolin-treated and untreated samples that were diluted in lysis buffer to vary the total protein content; the volume of lysis buffer was kept constant for all data points. (**d**,**e**) Forskolin-treated HT-29 lysates were assayed in the presence of various concentrations of H89 (**d**) and PKItide (**e**). Plotted values indicate the mean ± s.e.m. for triplicate measurements.

detected MK2 activation in CHO cell lysates treated with tumor necrosis factor (**Supplementary Methods** and **Supplementary Fig. 6** online). This activation correlated with the results of a radioactive MK2 assay[2] and demonstrated that the assay could be used with diverse stimuli and cell types.

For PKA, no adequate antibody was commercially available that could be used to perform either a radioactive PKA assay or an immunodepletion. Therefore, we used two mechanistically distinct PKA inhibitors to assess the selectivity of the PKA-S3 kinase assay: H89, a small molecule ATP-competitive inhibitor, and PKItide, the active fragment of a PKA-specific inhibitor protein[33]. PKA-S3 activity was abolished in a dose-dependent manner by both inhibitors (**Fig. 4d**,**e**). The calculated IC_{50} values (3.6 μM for H89 and 0.91 nM for PKItide) match those for the inhibition of recombinant PKA activity measured under identical conditions (**Supplementary Fig. 7** online). This inhibition data provides strong evidence that the PKA-S3 assay is highly preferential for monitoring target PKA activity in crude cell lysates.

The multiplex Akt-MK2-PKA kinase assay

To illustrate how these chemosensors can be used in a multiplex assay format, we examined Akt, MK2 and PKA signaling responses to two growth factors, insulin and EGF. HT-29 cells were treated with saturating concentrations of insulin or EGF and lysed in triplicate at various time points between 0 and 2 h. These lysates were then analyzed for Akt, MK2 and PKA activity with their respective kinase assays, and activation levels were calculated by normalizing to the 0-min data. For each growth factor, 63 independent activity assays were performed in parallel with less than 170 μg cell lysate. By comparison, a similar quantitative radioactive assay[2] for Akt and MK2 would require at least 700 μg cell lysate and would not be possible for PKA.

The multiplexed three-kinase assay revealed qualitatively and quantitatively different responses to EGF (**Fig. 5a**) and insulin (**Fig. 5b**). EGF activated MK2 more potently and transiently. Akt was also activated transiently in response to EGF, but to a lesser extent, and PKA was not activated at all. In contrast, insulin activated Akt potently, and the response was sustained over the time course. MK2 was slightly activated in response to insulin, and again, PKA was not activated at all. The features of these time courses correlate with those reported for Akt[34,35] and MK2 (ref. 36; or the MK2 kinase p38; ref. 37) activity dynamics in other mammalian cell lines. To the best of our knowledge, there is limited evidence that either EGF or insulin activate PKA.

DISCUSSION

Sox-containing peptide substrates have several desirable properties as molecular chemosensors for Akt, MK2 and PKA activity. For biochemical studies, the ability to synthesize the product phosphopeptide enables careful characterization of the fluorescence behavior and substrate turnover. Akt-S1, MK2-S1 and PKA-S3 have similar kinetic parameters (**Table 1**) to the optimal peptides[27–29] for these kinases, which indicates that the sensing module does not affect specific activity. Most importantly, the several-fold fluorescence difference between substrate peptide and product phosphopeptide is critical for sensitively monitoring changes in kinase activity. A mere 1% turnover corresponds to a 5–10% fluorescence increase, which is easily measured under the prescribed conditions. Finally, the stable fluorescence of the Sox-Mg^{2+} complex permits a continuous assay, which obviates problems with the termination steps of endpoint assays.

For biological applications, the specificity of any peptide substrate must be validated amidst total cellular proteins. Here this issue was

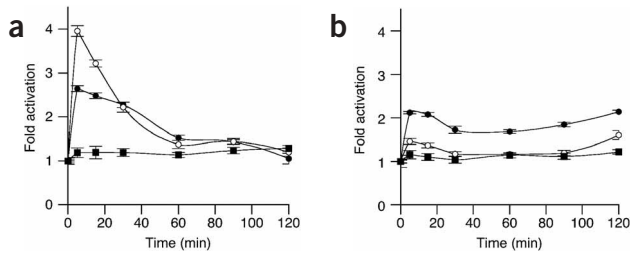

Figure 5 | The multiplex kinase assay reveals differential responses of Akt, MK2 and PKA in response to stimulation by EGF and insulin. (**a**,**b**) Akt-S1 (●), MK2-S1 (○) or PKA-S3 (■) kinase activities were measured in HT-29 lysates after stimulation by EGF (**a**) or insulin (**b**) for various amounts of time as described in Methods. Plotted values indicate the mean ± s.e.m. for triplicate measurements. Changes in kinase activity were normalized to the baseline (0 min) fluorescence slope.

addressed in several ways. First, the design of each substrate peptide was based on the optimal peptide sequences, implying that it is least likely to be outcompeted as a substrate for off-target kinases. Second, the Sox-based fluorescence readout is specifically attributable to Akt-S1, MK2-S1 or PKA-S3 phosphorylation, in contrast to other assays that measure bulk phosphotyrosine- or ^{32}P-labeled proteins. Third, assay selectivity was explicitly verified *in vivo* by small-molecule inhibitors and *in vitro* by immunodepletion. When inhibitor selectivity was questionable, another inhibitor that operates by a different mechanism was also used. These overlapping selectivity controls showed that the Akt-S1, MK2-S1 and PKA-S3 kinase activity assays are preferential, solution-phase assays for endogenous Akt, MK2 and PKA activities in cell lysates.

The homogeneous, fluorescence-based kinase activity assays reported here have several advantages over existing assays. The Sox-based chemosensors are uniquely quantitative in their ability to estimate enzymatic activity and amount of phosphorylated product generated from cell samples. The amount of lysate activity can easily be related to recombinant protein standards or normalized to untreated control lysates (**Fig. 5**). In addition, the assay is compatible with physiological concentrations of ATP. Together with the solution-phase format, this markedly increases the sensitivity of the assay. Whereas most immune complex kinase activity assays require 200–500 μg total cell protein[3], the kinase assays presented here can be used to make equivalent measurements at less than 100 μg (**Figs. 2b**,**d** and **3b**,**d**). This sensitivity is critical for applications in which cell samples are limited, such as high-throughput cell-based screening and clinical diagnostics. In addition, crude cell lysate assays eliminate several hours of manipulation, such as incubation and washing steps, before and after the *in vitro* reaction. During the kinase reaction, the 60 time points collected provide additional activity information and reduce error without extra experimental effort.

An important feature of these lysate-based assays is that selectivity can be improved by pharmacologically inhibiting kinases with overlapping substrate specificity. Because the relevant off-target enzymes will depend on the peptide chemosensor, kinase-by-kinase optimization will be needed to ensure maximum possible specificity. Although some residual off-target activity is inevitable (here $<$ 30% for Akt and 25% for MK2; **Figs. 2g** and **3g**), this singular limitation is outweighed by the many benefits of these fluorescent kinase activity assays, in that they are straightforward, rapid, continuous, nonradioactive, quantitative and sensitive. The format is conceptually similar to those of many fluorogenic protease assays[38] that, because of these same benefits, have found widespread use in numerous applications[39]. Finally, our work provides a general protocol for developing assays using other Sox-based chemosensors. As it is straightforward to multiplex chemosensors to measure several cellular kinase activities in parallel (**Fig. 5**), this assay platform is of immediate and expanding utility in drug discovery and molecular biology.

METHODS

Peptide synthesis. Fmoc-Sox-OH ((*S*)-2-amino-N^{α}-(9-fluorenylmethyloxycarbonyl)-3-(8-hydroxy-5-(*N*,*N*-dimethyl)quinoline-2-yl) propionic acid), was synthesized as previously described[20]. Peptides were synthesized as previously reported[19] and purified by reverse-phase HPLC (C_{18}) to $>$95% purity (see **Supplementary Methods** for peptide characterization). Concentrations of peptide stock solutions were determined by ultraviolet-visible (UV-Vis) spectrophotometry (the extinction coefficient (ε) of Sox is 8,247 M^{-1} cm^{-1} at 355 nm in 0.1 M NaOH with 1 mM Na_2EDTA) or by quantitative amino acid analysis.

Buffer recipes. Buffer A: 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 0.1 mM EGTA. Buffer B: 20 mM HEPES (pH 7.4), 1 mM DTT, 0.1% Brij-35, 1 mg/ml BSA. Buffer C: 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 30 mM NaF, 1 mM benzamidine, 2 mM EGTA, 100 μM Na_3VO_4, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml microcystin-LR. Buffer D: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 15 mM $MgCl_2$, 5 mM β-glycerophosphate, 1 mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM DTT.

Recombinant enzyme assays. Kinetic parameters were determined as previously described[19]. Recombinant enzyme was added to varying chemosensor concentrations (0.5–5 of K_M) in a fluorimeter at 30 °C. Assay conditions were as follows. Akt1: Buffer A with 40 ng Akt1 (Upstate; diluted 1:20 with Buffer B). MK2: Buffer A with 0.01% Brij-35, 0.1 mg/ml BSA, 20 ng MAPKAP kinase 2 (Upstate; diluted 1:10 with Buffer B). PKA: Buffer A with 8 ng PKA catalytic subunit (Calbiochem; diluted 1:80 with 20 mM Tris-HCl pH 7.5, 1 mM DTT, 0.15 mg/ml BSA). See **Supplementary Methods** for details of the data workup.

Cell stimulation and lysis: assay development and validation. Confluent 10-cm plates ($\sim$15 million cells) of HT-29 human colon epithelial cells (ATCC) were left untreated or stimulated with 500 ng/ml insulin for 5 min, 250 mM NaCl for 30 min, or 25 μM forskolin for 30 min and lysed in 300 μl Buffer C. Whole cell lysates were incubated on ice for 15 min, then clarified by centrifugation at 16,000*g* for 15 min at 4 °C. Protein concentrations of clarified extracts were determined with a bicinchoninic acid assay (Pierce). For inhibitor studies, cells were pretreated with various concentrations of wortmannin, LY294002 or SB202190 (Calbiochem) for 1 h before stimulation. The carrier concentration (0.1% DMSO) was maintained constant for all pretreatments.

Cell stimulation and lysis: insulin and EGF time courses. Time courses were performed as previously described[2]. Briefly, HT-29 cells were plated at 50,000 cells/cm^2 for 24 h. The cells were sensitized with 200 U/ml interferon-γ (Roche) for 24 h and then treated with 500 ng/ml insulin (Sigma) or 100 ng/ml EGF (Peprotech), each dissolved in 50% DMSO/50% water. At various time points after stimulation, clarified lysates were collected as described above.

Fluorescent kinase activity assays. Assay buffer (Buffer D with 4 μM PKC inhibitor, 4 μM calmidazolium (Sigma), 10 μM substrate peptide, 1 mM ATP (low metals grade; Calbiochem) and for Akt, 0.4 μM PKItide, 5 μM GF109203X (Calbiochem); for MK2, of 0.4 μM PKItide, 25 μM GF109203X; and for PKA, 5 μM GF109203X) was prepared in bulk at 30 °C and aliquoted into a glass 96-well plate (Zinsser with 500 μl or 300 μl well volumes, containing 250 μl or 150 μl reaction volumes, respectively) to ensure equal concentrations of the chemosensor. To begin each reaction, 7.5% (vol/vol) lysis buffer (Buffer C) or lysate (18.5 μl in 250 μl for Akt, and 11 μl in 150 μl for MK2 and PKA) was added

and the contents of each well mixed gently. During each reaction, 60 data points were collected. Akt-S1 activity was monitored over 60 min, typically with 93 μg lysate. MK2-S1 and PKA-S3 activities were monitored for 15 min, typically with 50 μg and 20 μg lysate, respectively. To quantify product formation, different turnover amounts (5%, 10%, 15%) were simulated in triplicate and lysis buffer was added as for a blank sample. For comparison with recombinant enzyme, different amounts (≥ 3) of the enzyme were added with lysis buffer to begin the reaction. For PKA inhibition studies, the indicated concentration of H89 or PKItide was included in the assay buffer. Chemosensor sensitivities were determined by comparing the absolute fluorescence of 10 μM substrate and 10 μM corresponding product phosphopeptides in triplicate under the optimized assay conditions. See **Supplementary Methods** for details of the data workup.

Radioactive Akt and MK2 activity assays. A microtiter-based kinase activity assay was performed as previously described[2] (**Supplementary Methods**).

Additional methods. Fluorescence instrumentation, and procedures for immunodepletion and western blot analysis are described in **Supplementary Methods**.

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

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Methods* website for details).

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