Supplementary Methods:

Peptide characterization data

Peptide	Sequence	Mol. formula	HPLC ret. time (min) [a]	*m/z* (*z*+) calcd	*m/z* found[d]
Akt-S1	Ac-Ala-Arg-Lys-Arg-Glu-Arg-Ala-Tyr-Ser-Phe-DPro-Sox-Gly-NH_2	$C_{79}H_{118}N_{26}O_{21}S$	31.0 [b]	600.6 (+3)	600.6
Akt-P1	Ac-Ala-Arg-Lys-Arg-Glu-Arg-Ala-Tyr-pSer-Phe-DPro-Sox-Gly-NH_2	$C_{79}H_{119}N_{26}O_{24}PS$	30.0 [b]	627.3 (+3)	627.6
MK2-S1	Ac-Ala-His-Leu-Gln-Arg-Gln-Leu-Ser-Ile-DPro-Sox-Gly-NH_2	$C_{69}H_{108}N_{22}O_{19}S$	31.4 [b]	791.4 (+2)	791.4
MK2-P1	Ac-Ala-His-Leu-Gln-Arg-Gln-Leu-pSer-Ile-DPro-Sox-Gly-NH_2	$C_{69}H_{109}N_{22}O_{22}PS$	30.1 [b]	831.4 (+2)	831.4
PKA-S3	Ac-Leu-Arg-Arg-Ala-Ser-Leu-DPro-Sox-Gly-NH_2	$C_{53}H_{86}N_{18}O_{14}S$	30.0 [b]	616.3 (+2)	616.2
PKA-P3	Ac-Leu-Arg-Arg-Ala-pSer-Leu-DPro-Sox-Gly-NH_2	$C_{53}H_{87}N_{18}O_{17}PS$	29.1 [b]	656.3 (+2)	656.2
PKItide	H_2N-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NH_2	$C_{80}H_{130}N_{28}O_{24}$	20.6 [c]	623.3 (+3)	623.1
PKC inhibitor	H_2N-Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val-NH_2	$C_{67}H_{119}N_{27}O_{15}$	24.0 [c]	386.5 (+4)	386.4

[a] C_{18}, Beckman Ultrasphere ODS, 5 μm, 150 x 4.6 mm; solvent A = water, 0.1% v/v TFA; solvent B = MeCN, 0.1% v/v TFA, [b]5 min 10% B followed by linear gradient 10-50% B over 30 min. [c]5 min 7% B followed by linear gradient 7-70% B over 30 min. [d]ESI-MS data was collected on a Applied Biosystems Mariner mass spectrometer.

Peptide stock solutions were prepared in ultrapure (18 MΩ) water and stored at 4°C for two months, or –20°C for longer periods.

Fluorescence instrumentation
Fluorimeter: Fluoromax 3 from Jobin Yvon (λ_{ex} = 360 nm, λ_{em} = 485 nm, slit widths = 5 nm, integration = 1 s). Cuvette: Starna Cells (16.100F-Q-10) 100 μl sub-micro cuvette, 1 cm path length.

Plate reader: HTS7000 Bio Assay Reader from PerkinElmer with 360 nm excitation filter (35 nm bandpass), 485 nm emission filter (20 nm bandpass), 40 μs integration, 3 flashes/well, 120 gain.

Improvement of the compatibility between lysis buffer and assay buffer
Initial attempts to analyze lysates led to precipitation and increased fluorescence emission due to light scattering. When precipitation was observed visually, centrifugation of the sample decreased emission at 400 nm back to the same level as a lysis buffer control and removed the contribution of light scattering to the fluorescence emission at 485 nm. Inclusion of 150 mM NaCl in the final assay mixture remedied the precipitation problem, as verified by a stable emission at 400 nm. Under these conditions, up to 150 μg of lysate protein in 7.5 vol% of the total reaction could be assayed without precipitation.

Reduction of nonspecific Akt-S1 kinase activity by GF109203X
Inclusion of 5 μM GF109203X in the assay buffer reduced non-specific Akt-S1 kinase activity by 75% (data not shown). Additional *in vivo* inhibition studies verified that atypical PKC isoforms, p70S6 kinase, and MAPKAP-K1 did not affect Akt-S1 fluorescence under the final assay conditions. To verify inhibition of atypical PKC isozymes, 5 μM Gö 6983 (Calbiochem) was included in the assay buffer. To verify inhibition of p70S6 kinase and MAPKAP-K1, HT-29 cells were pretreated for one hour

prior to insulin stimulation with upstream inhibitors of their respective pathways: 300 nM rapamycin (Calbiochem), to inhibit mTor, or 50 μM PD 98059 (Calbiochem), to inhibit MEK. No influence on measured Akt-S1 activity was observed in any of these assays when compared to the standard insulin-treated lysates.

Choice of 96-well plate and well volume
In polystyrene plates, significant absorption of assay components occurred, especially peptide (observed as a 10% fluorescence decrease over 20 min). A variety of traditional enzyme-linked immunosorbent assay (ELISA) blocking conditions were tried unsuccessfully. In glass plates, peptide adsorption is minimal and the assay is highly reproducible. In a plate format, longer path lengths improve the signal but require larger volumes per well. For efficient use of reagents, a compromise of 250 μl/well (Zinsser #3600500) or 150 μl/well (Zinsser #3600501) was reached, corresponding to a path length of 4 mm.

Data workup:
Recombinant enzyme assay
Reactions were monitored up to 10% turnover. Fluorescence slopes were converted to rates based on the absolute fluorescence of each chemosensor and corresponding product phosphopeptide. A Hanes plot was used to calculate K_M and V_{max}. Values reported are an average ± standard error for triplicate experiments.

Lysate assay
Fluorescence slopes were determined by a least-squares fit using KaleidaGraph (3.6.2, Synergy Software). This slope averages out scatter in individual fluorescence readings, as it is determined from 60 readings over the reaction time-course. To average out well-to-well variations in path length due to sample loading, each set of data points are normalized based on their starting values. Plotted values are an average ± standard error for triplicate experiments. For all samples except Figure 5, these triplicate measurements are separate reactions from the same cell population. For Figure 5, the results from 3 different populations were averaged for each data point. Average slopes of wells containing lysis buffer only were subtracted from average slopes of samples and the error propagated accordingly. Slight day-to-day variation in plate reader lamp intensity and in solution preparation were observed and thus comparisons were only made between samples run at the same time. Thus, the fluorescence slope in figures is not comparable between graphs. For comparisons of absolute fluorescence readings, wells were loaded carefully to obtain equivalent path lengths and measurements were performed in triplicate with multiple readings averaged for each well. Percent turnover was determined based on the fluorescence endpoint reading of each assay and a linear fit of absolute fluorescence vs. simulated turnover (see also Methods). Recombinant enzyme equivalents were determined by comparison of the fluorescence slope with a linear fit of the fluorescence slope vs. amount of recombinant enzyme (see also Methods).

Radioactive Akt and MK2 activity assays
Very briefly, Protein A or G microtiter strips were coated overnight with 10 μg/ml anti-Akt PH domain (Upstate) or anti-MK2 (Upstate) and washed three times with blocking buffer (1% BSA, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Trition X-100). Either 500 μg lysate for Akt and 200 μg lysate for MK2 was added for 3 h, and washed twice with Buffer D. Assays were conducted at 37°C in 60 μl Buffer D with 0.4 μM PKItide, 4 μM PKC inhibitor, 4 μM calmidazolium, ATP (10 μM for Akt and 25 μM for MK2), [γ-^{32}P]ATP (5 μCi for Akt and 2 μCi for MK2) and 10 μM Atkide or 10 μM MK2tide. The Akt assay was quenched after 30 min with 60 μl 75 mM H_3PO_4 and the MK2 assay was quenched after 15 min with 60 μl 20 mM EDTA. The reactions were prepared for scintillation counting as described[1].

Quenched-point fluorescence assay
Akt and MK2 were immunopurified from HT-29 lysates in multiples of nine as described for the radioactive activity assays. Assays were then conducted at 37°C in 60 μl Buffer D (see Methods) with 0.4 μM PKItide, 4 μM PKC inhibitor, 4 μM calmidazolium, 1 mM ATP and 10 μM Akt-S1 or 10 μM MK2-S1. 50 μl from three identical wells was transferred into a single well of the 96-well glass plate after 15 min for MK2 and 30 min for Akt at 25°C. Multiple fluorescence readings were averaged for each well. This resulted in triplicate measurements of 1250 μg lysate for Akt and 750 μg lysate for MK2 with a 4 mm fluorescence path length. A control reading of assay mixture that was not exposed to immunopurified kinase was subtracted from average sample readings. Values reported are the average ± standard error.

Chinese hamster ovary (CHO) cell maintenance and stimulation
CHO cells stably overexpressing the EGF receptor[2] were grown in DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin and 500 μg/ml G418. Cells were stimulated with 100 ng/ml EGF for 5 min or 100 ng/ml TNF (Peprotech) for 15 min and lysed as described for HT-29 cells (see Methods). 250 μg CHO cell lysate was used for the Akt assay, and 200 μg CHO cell lysate was used for the MK2 assay.

Immunodepletions
For Akt immunodepletions, 500 μg of insulin-stimulated HT-29 lysate was incubated with 4 μg anti-Akt PH domain (Upstate) or naïve mouse IgG (Santa Cruz) for two hours at 4°C on a rotating shaker. The immune complexes were precipitated with 40 μl Protein G sepharose beads (Amersham) for one hour with mixing at 4°C. For MK2 immunodepletions, 40 μl Protein G sepharose beads were incubated with 8 μg anti-MAPKAP kinase 2 (Upstate) or naïve sheep IgG (Santa Cruz) for one hour at 4°C on a rotating shaker. The beads were washed two times with 1 ml of blocking buffer (1% BSA in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Triton X-100) and then incubated with 200 μg NaCl-stimulated HT-29 lysate for three hours with mixing at 4°C.

Western blot analysis

To confirm immunodepletion, 100 μg of the starting lysate, kinase-immunodepleted lysate, and naïve-immunodepleted lysate were run on a 10% SDS-PAGE gel and transferred to PVDF (Millipore). The membrane was blocked with 5% nonfat skim milk in 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.1% Tween-20 and probed overnight with anti-Akt PH domain (Upstate), anti-Akt (Cell Signaling), or anti-MAPKAP K2 (Stressgen) at 1:1,000 dilution. The membranes were then probed with horseradish peroxidase conjugated anti-mouse or anti-rabbit secondary antibody (Amersham) at 1:10,000 dilution and visualized by enhanced chemiluminescence (Amersham) on a Kodak Image Station (Perkin Elmer). Bands were selected and quantified according to manufacturer recommendations. Additional rounds of immunodepletion substantially depleted Akt and MK2 from the naïve lysate (data not shown).

References

1. Janes, K. A. et al. A high-throughput quantitative multiplex kinase assay for monitoring information flow in signaling networks. *Mol. Cell. Proteomics* **2**, 463-473 (2003).
2. Harms, B. D., Bassi, G. M., Horwitz, A. R. & Lauffenburger, D. A. Directional persistence of EGF-induced cell migration is associated with stabilization of lamellipodial protrusions. *Biophys J*, in press (2005).