Methods

Protein kinases. Bovine PKA catalytic subunit was purchased from Sigma, *E. coli*-produced recombinant human CKII from Calbiochem (218701). Insect cell-produced recombinant active Akt was purchased from Upstate Biotechnology. Recombinant N-terminally hexahistidine-tagged PKC-θ and PKD were prepared in insect cells as describedS1. Active human His6-Erk2 was prepared by co-expression with active GST-MKK1 in *E. coli* and purified by immobilized metal affinity chromatography (Probond, Invitrogen) essentially as describedS2. Pim2 cDNA (MGC 8925) was obtained from ATCC and the coding sequence for the short isoform was subcloned by PCR into the mammalian expression vectors pEBG2 (to produce an N-terminal GST fusion construct) and pcDNA3 (in frame with an N-terminal FLAG epitope). Kinase inactive Pim2 was prepared by PCR-based mutagenesis of lysine 61 (short isoform numbering) to methionine (Quick Change mutagenesis kit, Stratagene). GST-Pim2 was produced in lipofectamine-transfected 293T cells, harvested 40 h post-transfection by washing cells once with cold PBS and extracting into lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO₄, 1 mM DTT, 1 mM PMSF, and 25 µg/mL each aprotinin, leupeptin and pepstatin A). Cleared lysates (10 min, 13,000 x g) were treated with on glutathione agarose beads (2 hr, 4°C), washed twice with lysis buffer, twice with wash buffer (50 mM HEPES, pH 8.0, 10 mM MgCl₂, 5 mM β-glycerophosphate, 0.1 mM Na₃VO₄) and eluted into wash buffer containing 20 mM glutathione and 10% glycerol. GST-TGFβRII cytoplasmic domain was expressed in 293T cells as for Pim2 above. Lysates (adjusted to 100 mM NaCl) were passed over a DEAE sephacel column. GST-
TGFβRII was eluted in lysis buffer containing 250 mM NaCl and purified on glutathione beads as above.

**Peptide libraries.** The peptide library was synthesized by Anaspec, Inc on a scale of 5 mg per peptide mixture. Degenerate positions were prepared by coupling isokinetic mixtures of the 17 naturally occurring amino acids excluding cysteine, serine, and threonine. Crude peptide mixtures were dissolved in DMSO and quantified by absorbance at 280 nm. Stock solutions were adjusted to 25 mM by adding the appropriate volume of DMSO and stored at −20°C. Working 0.5 mM aqueous stocks were prepared by diluting the DMSO stock rapidly into 50 mM HEPES, pH 7.4. To ensure representation of all amino acids at constant proportions within the library, aliquots of five of the libraries were sequenced by Edman degradation. The average molar proportion of each amino acid across all degenerate positions is shown in Figure S1. Aqueous solutions were aliquotted into 96-well polypropylene stock plates in 40 µl aliquots, sealed with adhesive foil, and stored at −20°C. Assays were performed by dispensing kinase buffer (see below) into 384-well plates (8µl per well). Peptides were transferred to assay plates (2 µl per well) from stock plates manually using a 96 slot pin replicator (VP Scientific). Reactions were initiated by adding kinase buffer containing 400 µM cold ATP with 0.1-0.2 µCi/µl γ-[32P]-ATP (4µl per well) and protein kinase (4µl per well diluted in kinase buffer. The final concentrations of the reaction components are 50 µM peptide and 100 µM ATP at a specific activity of 0.25-0.5 mCi/µmol. Between 20 and 100 ng of kinase were used per well, and kinase reactions were incubated at 30°C for 30 min to 8 hr (depending on the activity of the kinase). After incubation 2µl from
each well was spotted onto avidin-coated filter sheets (Promega SAM² biotin capture membrane) simultaneously using a 384 slot pin replicator (VP Scientific). Spots occupy approximately 16 mm² area and contain 100 pmol peptide; according to the manufacturer the membrane capacity for this area is 200 pmol. Membranes were washed three times with 10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.1% SDS, three times with 2 M NaCl, twice with 2 M NaCl, 1% H₃PO₄, and twice with water, dried, and exposed to a phosphorimager screen overnight. Buffers used for kinase assays were as follows. PKA: 50 mM Tris-HCl, pH 7.4, 20 mM MgCl₂, 0.5 mM β-glycerophosphate. Erk2 and Pim2: 20 mM HEPES, pH 7.4, 10 mM MgCl₂, and 1 mM DTT. Akt: 20 mM HEPES, pH 7.4, 10 mM MgCl₂, 10 mM DTT and 0.5 mM EGTA. CKII: 10 mM Tris-HCl, pH 7.4, 50 mM KCl, and 10 mM MgCl₂. PKD: 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT and 0.5 mM EGTA. PKC-θ: 50 mM Tris-HCl, pH 7.4, 8 mM DTT, 0.5 mM EGTA, 45 mM MgCl₂, 50 nM phorbol myristate acetate, 20 µg/ml phosphatidylserine. TGFβRII: 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 0.05% Tween 20.

**Kinase assays.** Individual peptide substrates were synthesized by the Tufts University Core Facility and purified by reversed phase HPLC. Kinetic parameters for Pim2 phosphorylation of the optimal Pimtide substrate were determined by incubating varying concentrations of peptide with 0.91 nM GST-Pim2 in Pim2 reaction buffer (above) containing 50µM ATP (with1 µCi/µl γ²P-ATP) for 5 min at 30°C. Aliquots (10 µl) of each reaction were spotted onto P81 phosphocellulose filters in triplicate and quenched in 0.42% H₃PO₄. Filters were washed four times in the same solution, dried, and radioactivity determined by scintillation counting. No phosphorylation was detectable
using kinase inactive mutant Pim2 protein (data not shown). Reaction rates for point-substituted Pimtide variant peptides were determined in a similar manner using 0.75 µM peptide. Pim2 phosphorylates Pimtide at 22 nmol/min per nmol enzyme under these conditions. Akt assays were performed similarly using 0.4 µg/ml Akt and 5 µM peptide substrate. The rate of phosphorylation of Aktide at this concentration is 3.8 nmol/min per nmol enzyme. Data shown are the average of three separate determinations.

**Supplemental Figure S1**

The chart shows the average molar proportion of each amino acid residue within the degenerate positions of the –3 Ala, Lys, Glu, Ile and Phe peptide mixtures. Molar proportions were determined by Edman sequencing of the libraries and normalizing the data to an average value of 1 within a given sequencing cycle. Tyr and Trp are not shown due to sequencing artifacts that prevent their accurate quantitation.

**Supplemental table S1**

Each worksheet provides quantitative data for the amount of radioactivity incorporated into each peptide mixture for one of the protein kinases evaluated. Incorporation of radioactivity was measured by phosphorimager using ImageQuant software (Molecular Dynamics). Data are normalized so that the average value for the 20 natural amino acids within a given position is 1, so that values greater than 1 represent positive selections. Selectivity values are therefore given relative to the other residues at the same position relative to the phosphoacceptor. Each worksheet includes a table summarizing positive selections (greater than 1.3) at each position. Note that oxidation is likely to make
quantitation of cysteine selections unreliable and that these selections should be regarded more qualitatively.

**Supplemental references**
