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A Quantitative Proteomics-based Competition Binding Assay to Characterize pITAM-Protein Interactions

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Abstract

Characterization of ligand-protein binding is of crucial importance in drug discovery. Classical competition binding assays measure the binding of a labeled ligand in the presence of various concentrations of unlabeled ligand, and typically use single purified proteins. Here we introduce a high-throughput approach to study ligand-protein interactions by coupling competition binding assays with mass spectrometry-based quantitative proteomics. Using a phosphorylated immunoreceptor tyrosine-based activation motif (pITAM) peptide as a model, we characterized pITAM-interacting partners in human lymphocytes. The shapes of competition binding curves of various interacting partners constructed in a single set of quantitative proteomics experiments reflect relative affinities for the pITAM peptide. This strategy can provide an efficient approach to distinguish specific interacting partners, including two signaling kinases possessing tandem SH2 domains, SYK and ZAP-70, as well as other SH2 domain containing proteins such as CSK and PI3K, from contaminants and to measure relative binding affinities of multiple proteins in a single experiment.

INTRODUCTION

Characterization of ligand-protein interactions is one important step in drug discovery. An understanding of the protein-drug or drug candidate binding affinity provides valuable insight into the functional roles of target proteins and directs evaluation of drug polypharmacology.¹ Traditional approaches to study ligand-protein interactions include ELISA, surface plasmon resonance, isothermal titration calorimetry, radioisotope- or fluorescence-labeled binding assays and so on,¹⁻⁴ all of which require previous knowledge of interaction candidates and in many cases the targeted protein needs to be purified for the assays.²⁻⁹ On the other hand, recent advances in mass spectrometry (MS)-based technologies such as immunoprecipitation¹⁰⁻¹² or tandem affinity purification¹³ enable the study of interacting proteins in a high throughput manner.¹⁴⁻¹⁸ The bait (ligand) is typically immobilized on a solid phase and the interacting proteins are purified from a complex matrix such as a cell extract. A major challenge in affinity purification-based approaches is contamination from non-specific binding proteins. There have been many efforts to address

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this problem, including the introduction of different epitope tags, the development of better washing and elution conditions, and the generation of a black list of commonly observed contaminating proteins.¹⁸⁻²⁰ In addition, other biochemical approaches, such as gene manipulation, can be used to verify interacting proteins. However, these experiments can be labor intensive and limited by the availability of proper biological systems.

Competition binding assays are commonly used to measure the binding affinity of a ligand with its receptor.^{21,22} In such an assay, the binding of a ligand labeled with a fluorescent or radioactive tag is typically measured at a single concentration in the presence of varying concentrations of an unlabeled, competing ligand. In order to measure accurate binding affinity, typically a single binding protein is applied in the assay. Recently competition binding experiments have been expanded to couple with quantitative proteomics to measure half maximal inhibitory concentration (IC₅₀) values of different tyrosine kinase inhibitors as the indication of kinase inhibitor potency.^{1,16} Sharma *et al*¹⁵ reported a more general strategy to determine dissociation constants of kinase inhibitors even if the affinity may change due to the modification and immobilization of the inhibitors. In this study, we applied the quantitative proteomics-based competition binding assay to probe interacting proteins of an immunoreceptor tyrosine-based activation motif (ITAM). SILAC (stable isotope labeling by amino acids in cell culture) was used for quantitative proteomics. We have estimated relative binding affinities of multiple proteins in a whole cell extract and the competition binding curve facilitates the distinction of specific binding proteins from non-specific ones.

An ITAM is a conserved sequence that exists in various proteins, most notably immune recognition receptors, that are associated with activation, survival, and differentiation of hematopoietic cells.²³ ITAMs, when phosphorylated on tyrosines, bind to Src Homology 2 (SH2) domains of effector proteins^{24,25} to trigger downstream signaling pathways. Therefore, knowledge of ITAM function, including the identification of phosphorylated ITAM (pITAM) interacting partners and the characterization of their binding affinities is critical to understanding the human immune system. In this report, specific interaction partners capable of binding an immobilized, phosphorylated ITAM were revealed using free pITAM peptide as the competitive binding reagent and SILAC as the quantitative method. Proteins identified include the well-known pITAM interacting proteins SYK and ZAP-70 as well as other novel interacting partners including CSK and PI3K. In addition, the competitive binding curves of these proteins were generated and used to verify the specificity of the interactions and to compare relative binding affinities. This strategy provides a powerful tool to identify specific ligand-interacting proteins in a high throughput approach.

EXPERIMENTAL PROCEDURES

Synthesis of the pITAM-Agarose Affinity Beads

The pITAM peptide CGGGDAVpYTGLNTRNQETpYETLKG (pY indicates a phosphorylated tyrosine) was derived from the γ chain of the high affinity IgE receptor (Fc ϵ RI). The peptide was synthesized using solid-phase Fmoc chemistry. One milliliter slurry of CarboxyLink Agarose beads (~16 μ mol amino groups) was washed 3 times with water, and then reacted with 32 μ mol 3-maleimidopropionic acid, 64 μ mol NHS (N-hydroxysulfosuccinimide) and 640 μ mol EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) in 2 mL of 200 mM MES buffer (pH6.0). The maleimide modified beads were then reacted with 0.16 to 16 μ mol of pITAM peptides in 5 mM tris(2-carboxyethyl)phosphine (TCEP)/PBS solution at room temperature for 30 min to cover 1-100% of the surface area on the beads. Finally, the beads were incubated in a 50 mM

cysteine solution for 2 h to block the unreacted maleimide groups. The modified bead was washed with 1 M NaCl and kept in PBS buffer for further use.

Preparation of Cell Lysate Samples

DG-75 human B lymphoma cells were grown to 50% confluency in RPMI-1640 media substituted with 10% heat-inactivated FBS, 1% sodium pyruvate, 0.5% streptomycin/penicillin, and 0.05% β -mercaptoethanol in 5% CO₂ at 37°C. For SILAC experiments, cells were grown to 50% confluency in SILAC RPMI-1640 media (Sigma) substituted with 10% dialyzed heat-inactivated FBS (Sigma), 1% sodium pyruvate, 0.5% streptomycin/penicillin, 0.05% β -mercaptoethanol, and either L-lysine and L-arginine for “light” samples, or ¹³C₆-arginine and ¹³C₆-lysine (Isotec) for “heavy” samples in 5% CO₂ at 37°C. Cells were grown for at least 6 divisions to ensure complete incorporation of the “heavy” amino acids (confirmed by mass spectrometry analysis). Before each set of experiments, “light” and “heavy” cell numbers were normalized.

Cells were washed with PBS, harvested, and frozen at –80°C. Cells were incubated with 1 mL of lysis solution, which contains 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1× Mini Complete protease inhibitor cocktail (Roche), 1 mM sodium orthovanadate, 1× phosphatase inhibitor mixture (Sigma) and 10 mM sodium fluoride for 20 min on ice. The cell debris was cleared at 13,200×g for 10 min, and the supernatant solutions containing soluble proteins were collected. The protein concentrations of cell lysates were determined using BCA assay (Pierce).

Affinity Pull-down and Competitive Binding

The pITAM-agarose beads (10 μ L slurry) were incubated with 200 μ L of cell lysate (1 mg) by rotating overnight at 4°C. The beads were washed three times with 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA and 1% NP-40 (pH 7.5). The captured proteins were eluted with 20 μ L SDS loading buffer and separated on a 12% SDS-PAGE gel for Western blotting analysis. For SILAC experiments, eight samples of “heavy” cell lysates (190 μ L, 200 μ g each) were incubated at 4°C for 1 h with 10 μ L of free pITAM peptides with concentrations of 800, 400, 200, 80, 40, 20, 8 and 0 μ M, respectively. Then a 10 μ L slurry of pITAM-agarose beads was added to each of the pre-incubated cell lysates. Similarly, another eight aliquots of 10 μ L slurries of pITAM-agarose beads were mixed with eight “light” lysate samples (190 μ L, 200 μ g each). After incubation at 4°C overnight, all beads were washed 3 times with the washing buffer. Then each batch of the beads incubated with the “heavy” lysate was mixed with one set of beads incubated with the “light” lysate. Finally all eight sets of beads were washed twice with the washing buffer.

On-beads Digestion and NanoLC-MS/MS Analysis

The beads were resuspended in 100 μ L of denaturing buffer (8 M urea in 100 mM NH₄HCO₃). The samples were then mixed with 4 μ L of 300 mM DTT and incubated at 37°C for 1 h to denature the proteins. Six microliters of 600 mM iodoacetamide solution was then added and the samples were incubated at room temperature for 1 h in the dark. The samples were diluted with 400 μ L of 100 mM NH₄HCO₃ and digested with 2 μ g of trypsin at 37°C overnight. The digested mixture was first acidified with 1% formic acid and then desalted with a 100-mg Sep-Pak C18 column (Waters).

The resultant peptide samples were dissolved in 20 μ L of 0.1% formic acid, and 8 μ L of solution was injected into an Eksigent NanoLC Ultra 2D system. The reverse phase C18 separation was performed using a C18 capillary column (75 μ m i.d.) packed in-house with 5 μ m C18 Magic beads resin (Michrom, 12 cm of bed length) on an Eksigent Ultra2D HPLC. The mobile phase buffer consisted of 0.1% formic acid in ultra-pure water with the eluting

buffer of 0.1% formic acid in 100% CH₃CN run over a shallow linear gradient from 2% CH₃CN to 35% CH₃CN over 90 min with a flow rate of 300 nL/min. The electrospray ionization emitter tip was generated on the pre-packed column with a laser puller (Model P-2000, Sutter Instrument Co.). The Eksigent Ultra2D HPLC system was coupled online with a high resolution hybrid linear ion trap orbitrap mass spectrometer (LTQ-Orbitrap Velos; Thermo Fisher). The mass spectrometer was operated in the data-dependent mode, in which a full-scan MS (from *m/z* 300-1700 with the resolution of 30,000 at *m/z* 400) was followed by 7 MS/MS scans of the most abundant ions. Ions with charge state of +1 were excluded. The mass exclusion time was 90 s.

Data Acquisition and Analysis

The LTQ-Orbitrap raw files were searched against IPI *homo sapiens* database with no redundant entries (67, 250 entries; human International Protein Index v.3.64) using SEQUEST algorithm on Proteome Discoverer (Version 1.2; Thermo Fisher). DTA files were generated using Proteome Discoverer with the minimum ion threshold of 15 and absolute intensity threshold of 50. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.8 Da. Search criteria included a static modification of cysteine residues of +57.0214 Da and a variable modifications of +15.9949 Da of methionine oxidation. Searches were performed with full trypsin digestion and allowed a maximum of two missed cleavages on the peptides analyzed from the sequence database. False discovery rates (FDR) were set at 1% for each analysis. Proteome Discoverer generates a reverse “decoy” database from the same protein database, and any peptide passing the initial filtering parameters that were derived from this decoy database are defined as false positive identification. The minimum cross-correlation factor (Xcorr) filter was re-adjusted for each individual charge state separately in order to optimally meet the predetermined 1% target FDR.

For SILAC experiments, an additional static modification (+6.020 Da) of heavy isotopes on lysines or arginines was also included. Quantitation and statistics analyses were performed using Proteome Discoverer (Version 1.2). Only unique peptides with a mass precision below 2 ppm were used for quantitation. The distributions of Heavy/Light ratios of quantified proteins were generated, and the standard deviations were calculated from the distributions. Proteins with a Heavy/Light ratio larger than the three standard deviations were considered significantly changed (statistics analyses were performed simultaneously by the Proteome Discoverer software).

RESULTS AND DISCUSSION

Rationale of the quantitative-proteomics based competition binding assay

An ITAM is a conserved sequence (YXX(L/I)X₆₋₈YXX(L/I)) of 14-16 amino acids found in the cytoplasmic tails of certain cell surface proteins of the immune system.^{10,26,27} ITAMs, when phosphorylated, are known to bind Src Homology 2 (SH2) domains in proteins (Figure 1a) and trigger downstream signaling events. Examples include the B cell Receptor for antigen (BCR) and high affinity IgE receptor (FcεR1) activation pathways. Phosphorylated tyrosine peptides have been explored to study phosphorylation-dependent peptide-protein interaction due to its importance in regulation of signaling pathway²⁸⁻³⁰. To identify novel interacting partners of pITAMs based on competition binding and quantitative proteomics, we generated a peptide containing a doubly phosphorylated ITAM derived from the FcεRI γ chain containing a triglycine linker as a spacer and a terminal cysteine residue for convenient immobilization. The modified pITAM peptide was immobilized onto agarose resin by a two-step reaction. The surface of agarose beads was modified with maleimide

functional groups in the first step, and in the second, the modified ITAM peptides were immobilized via the maleimide-thiol reaction (Figure 1b).

The competition binding assay was performed by adding different amounts of the free pITAM peptide into the cell lysate before affinity purification. The free pITAM peptide thus first binds to any interacting proteins to compete for binding with the immobilized pITAM peptide. The level of specific binding of proteins to the immobilized pITAM was determined in the presence of a range of concentrations of free pITAM in solution. Proteins binding to the pITAM-agarose were eluted, identified, and quantified by MS. Proteins participating in non-specific binding interactions should not be affected by the addition of free pITAM peptide. On the other hand, highly specific binding proteins will be reduced in level by the presence of free pITAM.

Figure 2 illustrates the strategy for the competition binding assay using SILAC-based proteomics. DG75 cells used in this experiment were cultured in heavy and light isotopically labeled media, respectively. No competing pITAM peptide was added to the affinity purification experiment using cells grown in media with light isotopes. On the other hand, 8 different concentrations (0 to 40 μ M) of free pITAM peptide were spiked into the cell extracts in experiments using cells grown in heavy media. The heavy and light isotope labeled protein samples were then mixed and digested on-beads for mass spectrometric analysis. Each peptide from a putative pITAM interacting protein has two mass spectrometry signals, i.e., heavy and light. The relative ratios of protein-derived peptides between each pair of heavy and light signals was determined as a function of free pITAM concentration, generating a plot, similar to a dose-response curve in a classical competition binding assay. In addition to the identification of novel interacting proteins, we also determined their relative binding affinities for pITAM peptides in one series of mass spectrometry-based competition binding experiments. Typically, the binding properties of only a single protein can be obtained when performing conventional competition binding assays.^{18,31}

Identification of SYK as a highly specific ITAM-binding protein

SYK is a well-known pITAM interacting protein in human cells with its tandem pair of SH2 domains³². For example in B cells, cross-linking of the BCR leads to the phosphorylation of ITAM tyrosines to recruit kinase SYK to the receptor leading to its activation. The recruitment of Syk to a phosphorylated ITAM is critical for signaling from many additional immune recognition receptors, including Fc γ and Fc ϵ receptors and natural killer cell activating receptors.¹⁷ Therefore, the interaction between a pITAM motif and SYK was used here to illustrate the specificity of the proteomics-based competition binding assay. The agarose resins with immobilized pITAM peptide were tested for recovery of SYK from cell lysates of DG75 pre-treated with or without pervanadate, a tyrosine phosphatase inhibitor that activates Syk. As shown in Figure 3a, pITAM affinity beads can successfully purify SYK protein from both treated and non-treated cell lysates in high yield, while the Western blot of control beads (agarose resins without pITAM) show little signal. Previous investigation on the binding specificity of pITAM and ITAM showed that ITAM without phosphorylation did not interact with the kinases such as Syk²⁵. Therefore, phosphatase inhibitor cocktails were used to prevent the dephosphorylation by active phosphatases in the lysate. We also carried out the affinity pull down experiments without phosphatase inhibitor in the lysates. Surprisingly, no SYK and other specific interaction protein could be identified, which indicates the effect of active phosphatases in cell lysates and demonstrated the specific recognition by phosphorylated ITAM. Many of the known pITAM interacting proteins are phosphorylated on tyrosine. Therefore we performed Western blotting with 4G10 anti-phosphotyrosine antibody to characterize the total captured proteins. As expected, few proteins were observed binding to the control beads, while many tyrosine

phosphorylated proteins were observed binding to the pITAM-agarose beads (Figure 3b). Pervanadate treatment significantly elevated tyrosine phosphorylation in the cell, leading to higher signaling of phosphotyrosine containing proteins in the pITAM-purified sample.

The results from the proteomics-based competition binding assay confirmed strong interactions between the pITAM and SYK. SYK protein was identified in all the SILAC experiments repeatedly and the dose-response curve based on these experiments (Figure 4) indicated that the amount of bound SYK was strongly reduced in response to increasing amounts of free pITAM peptide added to the cell lysates. Western blotting experiments using antibodies against SYK protein verified the MS identification and quantification results (Figure 5).

Identification and quantification of novel ITAM interacting proteins

Novel ITAM interacting proteins were identified according to the following rules. First, the proteins had to be identified multiple times from the eight pull-down experiments with at least two peptides at FDR less than 0.01. Second, the MS signals of these peptides in the heavy isotopically labeled samples (in the presence of free pITAM) were less than those in the light samples (without free pITAM). Finally, the ratios between the heavy and light signals formed a typical dose-responsive curve. Under these rules, six additional pITAM interacting proteins were identified. Binding affinity curves were generated and are shown in Figure 4 and Supplementary Figure S1.

The plots of relative ratio of bound proteins as a function of free pITAM concentration (Figure 4) illustrates representative binding curves of three groups of proteins identified from the pull-down experiments. These include peroxiredoxin representing a non-specific binding protein, strong pITAM interacting proteins SYK and ZAP-70, and weaker pITAM binding proteins PIK3R2 and CSK. A complete list of the proteins identified and quantified can be found in the supplementary data. Several high abundant non-specific binding proteins (e.g., peroxiredoxin, elongation factor and ribosomal protein) did not show dose-dependent competition. ZAP-70 and SYK have similar binding affinities because they belong to the same kinase family³³ and both have tandem SH2 domains. While ZAP-70 is usually present in T cells,³⁴ it is also expressed in a number of B cell lymphomas where it is associated with a poor patient prognosis. With one SH2 domain, CSK shows a weaker binding affinity to pITAM. It is known that CSK interacts with SYK,³⁵ and the possibility cannot be excluded that, instead of directly interacting with the pITAM, CSK formed a complex with SYK, which interacted with pITAM. To address this issue, we performed affinity enrichment with SYK-deficient DT40 B cells in parallel with cells in which the expression of SYK was restored by transfection with plasmids coding for wild-type SYK. CSK protein could be easily identified as a pITAM-binding protein by MS from both cell lines, which confirms that CSK is the direct binding partner of pITAM (data not shown).

In addition, several members from the PI3K family and a protein INPP5D/SHIP, which negatively regulates the PI3K pathway, were also identified as novel interacting partners of pITAM. The binding curve of protein PIK3R2, the p85 regulatory subunit of PI3K, is shown in Figure 4 and the curves for the other binding proteins are shown in Supplementary Figure S1. While PIK3R2 also contains two SH2 domains, its binding affinity is not nearly as strong as that of SYK or ZAP-70. This is likely explained by the topology of PIK3R2, which does not favor binding between the pITAM peptide and the two SH2 domains simultaneously.

Can proteomics-based competition binding assay measure ligand-protein binding constants in cell extracts?

Besides the benefit of differentiating specific binding proteins from others, the strategy is appealing with the possibility of measuring multiple ligand-protein binding constants, more commonly the dissociation constant K_d , in the same experiment. Cheng-Prusoff equation relates affinity (dissociation) constants and IC_{50} ¹⁵ and under specific conditions, the dissociation constant (K_d) is approximately equal to IC_{50} and thus IC_{50} values can be compared in different experiments. Based on this feature, Bantscheff *et al*¹ quantitatively assessed more than 100 protein kinases with multiple kinase inhibitors in leukemia cell extracts. By limiting the amount of immobilized beads to minimum, they proposed to measure IC_{50} as K_d between inhibitors and targeted kinases. However, data obtained with this approach considerably vary from IC_{50} values measured *in vitro* with purified kinases. This disparity is likely due to the fact is that free ligands and immobilized ligands do not have the same affinity to targeted proteins. In a classical competition binding assay where the competition is between radio-isotopic labeled ligand and unlabeled ligand under the homogenous condition, it assumes the target protein has identical affinity for the competing ligands, whereas the competition in the proteomics-based approach is between free ligand and ligand on the solid phase. Due to steric effect, it is conceivable that the affinity of immobilized ligand is different from that of free ligand to most proteins. To account for different affinities due to modification and immobilized of ligands, Sharma and co-workers¹⁵ devised a strategy by coupling two sets of SILAC-based quantitative proteomic experiments. First, by incubating cell extracts with immobilized kinase inhibitor resin sequentially, it calculated the K_d of the immobilized inhibitors. Second, by performing a similar SILAC-based competition binding experiments, it used the Cheng-Prusoff equation to calculate K_d of the “free” (unmodified) kinase inhibitors. In all these experiments, in order to measure an accurate K_d values (typically in nM to μ M range) using the IC_{50} value, the amount of the ligand immobilized on the solid phase needs to be optimized to keep its concentration in similar or lower range of concentrations. This condition may result in low efficiency in affinity purification due to the small amount of beads and low level of binding ligand used in the proteomics experiment. Furthermore, it assumes ligand is not depleted in the competition assay. However, in the whole cell extract or complex mixture there are potentially multiple proteins that can bind to the ligand and may indeed lead to depletion of the ligand. The competition and interactions among these proteins can further complicate the analysis, a phenomenon well recognized by a previous study using reactive probes for activity-based competition binding experiments.³⁶ To illustrate these issues, we investigated the two binding proteins, SYK and CSK, in the ITAM system. Western blotting results indicate that both signals of SYK and CSK decreased due to the competition of free ITAM (Figure 5a). However, the signals did not drop until 40 μ M of free ITAM was added to the lysate, which is much higher than the literature reported K_d value of ITAM to SYK, likely due to excess amount of ITAM-bound beads in the experiment (beads were immobilized with excess ITAM peptides), indicating the condition cannot be used to measure K_d based on the IC_{50} value. The immobilized ITAM concentration was thus decreased to 1% which corresponds to low μ M in concentration, and the IC_{50} of CSK dropped to in between 400 nM and 4 μ M (Figure 5b). But the IC_{50} of SYK still remains high. To avoid potential ligand depletion, we decreased the amount of DG-75 cell lysate from 1mg to 200 μ g and both IC_{50} of the two proteins are decreased to low μ M level, which are closer to the values reported in the literature (Figure 5c). However, mass spectrometric analyses were not sensitive enough not able to construct a competitive binding curve due to low amount of ITAM beads and lysate used in the experiment (data not shown). The experiments demonstrate that in order to accurately measure multiple ligand-protein binding constants, the amount of immobilized ligand and whole cell extract needs to remain at minimum, both of which will greatly affect

the sensitivity of quantitative measurement in the experiment. Future instrumental advances with high sensitivity may facilitate the measurement.

CONCLUSION

An integrated quantitative proteomics method was developed to identify and verify ITAM interacting proteins from a complex sample, and characterize the relatively binding affinities of interacting partners. The method is developed by coupling a modified competition binding assay with the SILAC-based quantitative proteomics approach. Specifically binding proteins were successfully identified by constructing corresponding dose-response curve. As a high throughput approach to identify multiple interacting proteins and verify the specific bindings by quantitation results, this strategy can be widely applied in various systems to solve the problem of distinguishing specific protein-ligand interactions from the non-specific binding proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BCR	B cell receptor
CSK	c-Src tyrosine kinase
FDR	False discovery rate
IC50	Half maximal inhibitory concentration
ITAM	Immunoreceptor tyrosine-based activation motif
pITAM	Phosphorylated Immunoreceptor tyrosine-based activation motif
SILAC	Stable isotope labeling with amino acids in cell culture
PI3K	Phosphoinositide 3-kinase
SH2	Src Homology 2
SYK	Spleen tyrosine kinase
ZAP-70	Zeta-chain-associated protein kinase 70 kDa

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Figure 1. Synthesis of ITAM-agarose beads and the binding of ITAM with tandem SH2 domain. a). Phosphorylated ITAM peptide strongly binds to proteins containing tandem SH2 domains. Lowercase p illustrates phosphorylated tyrosine residues. The sequence was derived from the γ subunit of the high affinity IgE receptor (Fc ϵ RI γ). b). Immobilization of ITAM peptides to agarose beads. CarboxyLink Agarose beads were reacted with 3-Maleimidopropionic acid, and then ITAM peptides were immobilized onto agarose beads via maleimide-thiol reaction.

Figure 2. Schematic representation of the SILAC-based competition binding assay. See text for details.

Figure 3.

Western Blotting of lysate and immunoprecipitation results using anti-SYK antibody (a) or anti-phosphotyrosine (b), with ITAM-agarose beads and control beads (agarose without ITAM) in DG-75 cells with and without pervanadate treatment.

Figure 4.
Representative competition binding curves constructed from the proteomic experiments.

Figure 5.

Condition optimizations to measure IC₅₀s of interacting proteins. a). Agarose resins were immobilized with excess amount of ITAM peptide. One milligram of DG75 cell lysate was used in affinity purification. b). Agarose resins were immobilized with 1% equivalent of ITAM peptide. The same amount of lysate (1 mg) was employed. c). Different amounts of whole cell extracts for the competition binding assay. The amount of ITAM peptides on the resin is the same as in b).