Materials and Methods

Chemicals were generally obtained from Aldrich, except for piperidine (Advanced Chemtech), protected amino acids, amino acid derivatives, HOBt, and BOP (Advanced Chemtech and Bachem California), Rink resin, Wang resin, and Tentagel resin (Advanced Chemtech). 2-(4'-(iodoacetamido)anilino)naphthalene-6-sulfonic acid (IAANS) was obtained from Molecular Probes. Biotinyl-ε-aminocaproyl-EPQpYEEIPIYL was purchased from Bachem California. SH2-GST fusion proteins (Lck (120-226), Fyn (145-247), PLCγ1 (548-659), PI-3Kp85α (333-430), and GRB2 (54-164)) and polyclonal rabbit anti-GST antibody were purchased from Santa Cruz Biotechnology. Horse radish peroxidase-conjugated mouse anti-rabbit antibody, peroxidase substrate (1-Step Turbo TMB-ELISA, trimethylbenzidine), steptavidin coated 96-well plates, and Slide-A-Lyzer 10K MWCO dialysis slide cassettes were purchased from Pierce. Solvent resistant MultiScreen 96-well filter plates and the MultiScreen 96-well filter plate vacuum manifold were purchased from Millipore.

Peptide Synthesis

All peptides, except biotinyl-ε-aminocaproyl-EPQpYEEIPIYL, were synthesized on an automated peptide synthesizer (Advanced Chemtech model ACT90) using a standard Fmoc solid phase peptide synthesis protocol. Rink amide and Wang acid resins were employed for the preparation of peptides containing amide and free acid C-terminii, respectively. Crude peptides were purified on a preparative HPLC using three Waters radial compression modules (25 x 10 cm) connected in series. Purified peptides were further characterized by mass spectrometry.

The AANS-labeled probe peptide was prepared by dissolving FTATECQpYEEIP into 100 mM sodium phosphate (pH 7.8) with 1 mM DTT at concentration of 4 mg/mL. The solution was stirred for 30 min to ensure complete reduction of cysteine side chain. An aliquot of IAANS (100 mM in DMF) stock solution was added to the peptide solution to yield a final concentration of 6

mM IAANS. The reaction proceeded for 5 hr at room temperature in the dark. The labeled peptide was purified via preparative HPLC and characterized by mass spectrometry. The concentration of the AANS-labeled peptide stock solution was determined by both weight and UV spectrophotometry using an extinction coefficient of 26000 M^{-1} cm^{-1} at 329 nm in methanol.

Synthesis of Peptide/Nonpeptide Conjugate Library

Cystamine dihydrochloride (10 eq, 2.25 g) was added to a mixture of Tentagel S COOH resin (130 μm, 5 g, 0.2 mmol/g), BOP (1.2 eq, 530 mg), HOBt (1.2 eq, 152 mg), and NMM (30 eq, 2.8 mL) in 20 mL DMF and subsequently shaken overnight at room temperature. The free amine substitution level was 0.01 mmole/g. This low substitution level is ideal for our purposes since this not only ensures a higher coupling yield but, in addition, larger quantities of resin (with greater weight accuracy) can be subsequently introduced into the 96 well plates (*vide infra*). The peptide FmocNH-pYE(t-butyl)E(t-butyl)I was synthesized on the cystamine-substituted Tentagel resin using an Fmoc solid phase peptide synthesis protocol. After deprotection of the N-Fmoc group, the resin was extensively washed and subsequently dried in vacuum. The peptide-bound resin was distributed in 5 mg quantities into each well of solvent-resistant 96-well filter plates. In addition, each well contained a carboxylic acid-containing compound (400 eq, 20 μmol), BOP (200 eq), HOBt (200 eq), and NMM (1000 eq) in 100 μL DMF. A total of 900 different carboxylic acids were employed. The plates were shaken overnight and then each well was subjected to a series of wash steps (3 x 200 μL DMF; 3 x 200 μL water; 3 x 200 μL DMF; 3 x 200 μL CH_2Cl_2) using a 96-well filter plate vacuum manifold. The Glu t-butyl side chain protecting groups were subsequently deprotected with 50% trifluoroacetic acid in CH_2Cl_2 (2 hr) followed by a second series of wash steps (2 x 200 μL CH_2Cl_2; 2 x 200 μL DMF; 2 x 200 μL water; 200 μL 10% NMM in CH_2Cl_2; 2 x 200 μL 50 mM Tris pH 7.5). The peptide-nonpeptide conjugates were cleaved from the disulfide-containing resin with 10 mM DTT in Tris buffer (1 x 200 μL for 1 hr; 2 x 150 μL for 1 hr each) and filtered into a receiving set of 96-well plates using the vacuum manifold (final volume: 500 μL). The efficiency of acid coupling, peptide cleavage from the resin

with DTT solution, and purity of the peptide/nonpeptide conjugates were assessed with several ligands (acetyl-, 7-hydroxycoumarin-4-acetyl-, 2-bromo-dimethoxybenzoyl-, and 4-nitrohippuryl-). No free N-terminus peptide was detected and over 90% of total ligand was cleaved from resin with first DTT wash step. The final two DTT washings removed the residual resin-bound material. Compound purity was greater than 90% as assessed by HPLC and the HPLC-purified compounds (i.e. removal of Tris buffer and DTT) were characterized by electrospray mass spectrometry.

Screening of the Peptide/Nonpeptide Conjugate Library

An enzyme-linked immunosorbant assay was employed to screen the library for SH2 affinity. 100 μL of biotinyl-ε-aminocaproyl-EPQpYEEIPIYL (10 ng/mL in 50 mM Tris, 150 mM NaCl pH 7.5) was added to each well of streptavidin-coated 96-well microtiter plates. The plates were shaken overnight at 4 °C and rinsed with "TBS" (50 mM Tris, 150 mM NaCl, pH 7.5, 3 x 200 μL). Each well was then blocked with 100 μL of a solution containing 2% BSA and 0.2% Tween 20 in TBS (1 hr at 37 °C). The wells were then rinsed with 4 x 200 μL of a standard "BSA-T-TBS" solution (0.2% BSA, 0.1% Tween 20, TBS). A 50 μL solution of the peptide/nonpeptide conjugate (100 nM, in BSA-T-TBS) from the library and a 50 μL solution of the Lck SH2-GST fusion protein (10 ng/mL, in BSA-T-TBS) were added in each well and the plate was shaken for 1 hr at room temperature. The solutions were removed and each well rinsed with 4 x 200 μL BSA-T-TBS. 100 μL of polyclonal rabbit anti-GST antibody (100 ng/mL in BSA-T-TBS) was then added to each well and incubated for 1 hr at room temperature. Following subsequent washing steps with BSA-T-TBS (4 x 200 μL), 100 μL of horse radish peroxidase-conjugated mouse anti-rabbit antibody (200 ng/mL in BSA-T-TBS) was added to each well and subsequently incubated for 1 hr at room temperature. After a series of final wash steps (4 x 200 μL BSA-T-TBS; 2 x 300 μL TBS), 100 μL of peroxidase substrate (1-Step Turbo TMB-ELISA, trimethylbenzidine) was added to each well and incubated for 5 - 15 min. 100 μL of 1 M sulfuric

acid solution was introduced to stop the peroxidase reaction and absorbance was measured at 450 nm with a plate reader.

Determination of K_d Values:

Competition Assay Versus AANS-Labeled Peptide

The AANS-labeled peptide [FTATEC(AANS)QpYEEIP] changes in emission intensity (approximately 8-fold) upon binding to either the Lck or Fyn GST-SH2 fusion protein. The K_d for this peptide was determined by measuring the fluorescence change upon adding varying concentrations of GST-SH2 fusion protein into a solution containing 100 nM AANS-labeled peptide. The K_ds for non-fluorescent peptide/nonpeptide conjugates were determined by a competitive binding fluorescence assay. Competition experiments were performed by measuring fluorescence changes upon titrating nonfluorescent SH2-targeted compounds into a solution containing 100 nM Lck or Fyn GST-SH2 fusion protein and 1 μM AANS-labeled peptide. All samples were prepared in a buffer containing 50 mM Tris, 150 mM NaCl, and 1 mM DTT at pH 7.5. Fluorescence measurements were performed at 25 °C in 1 cm pathlength cuvettes. The excitation wavelength was 330 nm and emission was monitored at 450 nm. The following equation was used for the determination of K_L (i.e. K_D for the peptide/nonpeptide conjugate for the SH2 domain):

$$K_L = \frac{K_p[RP]}{[P]_T - [RP]}\left(\frac{[L]_T}{[R]_T - \frac{K_p[RP]}{[P]_T - [RP]} - [RP]} - 1\right)$$

where $K_L = K_D$ of peptide-nonpeptide conjugate for SH2 domain, $K_p = K_D$ of AANS-peptide probe for SH2 domain, $[P]_T$ = total AANS-peptide probe concentration, [RP] = concentration of SH2/AANS-peptide probe complex, $[L]_T$ = total peptide/nonpeptide concentration, $[R]_T$ = total SH2 domain concentration.

Determination of K_d Values:

The Equilibrium Dialysis Method

Both 7-hydroxycoumarin-4-acetyl-pYEEI-NH_2 (**5**) and 7-methoxycoumarin-4-acetyl-pYEEI-NH_2 (**11**) are highly fluorescent compounds that exhibit no change in fluorescence upon coordination to the SH2 domains of either Lck or Fyn. Therefore, the K_ds for the SH2 complexes of **5** and **11** were determined via equilibrium dialysis. All samples were prepared in a buffer containing 50 mM Tris, 150 mM NaCl, and 1 mM DTT at pH 7.5. Slide-A-Lyzer dialysis slide cassettes (Pierce, 10K MWCO, 0.1-0.5 mL capacity) were employed and contained 100 - 200 nM Lck GST-SH2 (with peptide **5**), 1 μM Lck GST-SH2 (with peptide **11**), or 1.5 μM of PLCγ1, PI-3Kp85α, and GRB2 GST-SH2 fusion proteins (with peptide **5**). The K_D for peptide **9** (5 - 10 μM) with the Lck GST-SH2 (50 nM) was obtained via the competitive displacement of the coumarin derivative **5** (500 nM). In all cases, the slide cassettes contained a final volume of 250 μL. The cassettes were placed in a beaker containing a volume of buffer solution (50 mM Tris, 150 mM NaCl, and 1 mM DTT at pH 7.5) that was at least 400-fold greater than that of the sample volume in the dialysis slide cassette. As a consequence, concentrations of non-SH2-bound coumarin-containing peptide and non-SH2-bound nonfluorescent peptide/nonpeptide conjugate were held constant in the dialysis slide cassette over the course of the experiment. Equilibrium dialysis experiments were performed over a period of 6 hr and maintained at 4 °C. Differences in fluorescence between the solution in the slide cassette and that in the beaker were measured. The excitation wavelength employed for both 7-hydroxycoumarin-4-acetyl-pYEEI-NH_2 and 7-methoxycoumarin-4-acetyl-pYEEI-NH_2 was 330 nm. Emission was monitored at 460 nm for the 7-hydroxy derivative and 392 nm for the 7-methoxy analog. The following equation was used for the determination of K_L (i.e. K_D for the peptide/nonpeptide conjugate for the SH2 domain):