SUPPLEMENTAL MATERIAL

REGULATION OF SYK BY PHOSPHORYLATION ON SERINE IN THE LINKER INSERT

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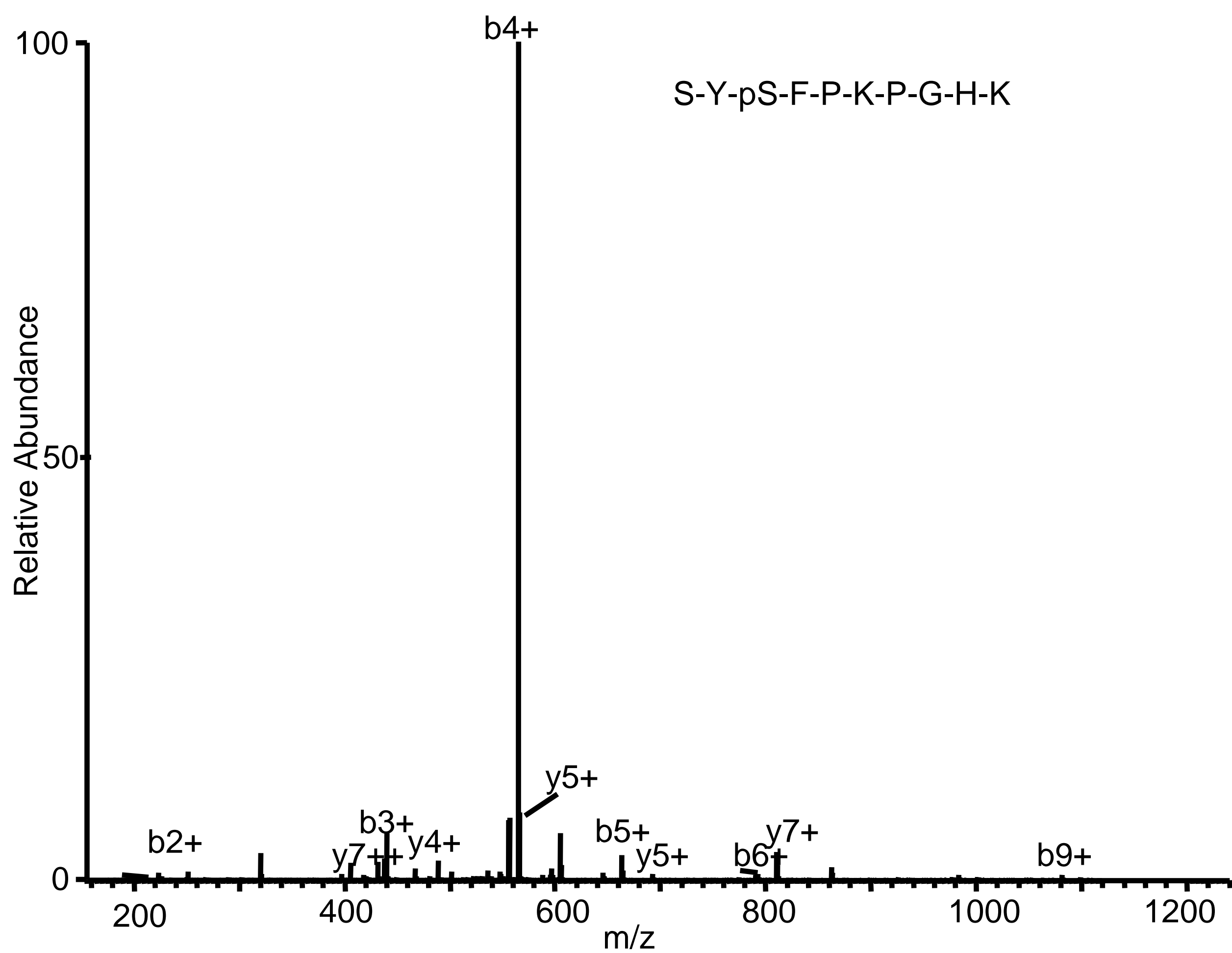
EXPERIMENTAL PROCEDURES

Sample preparation—Beads containing biotinylated peptides were washed 3 times with lysis buffer and three times with H_2O. Proteins were eluted in 100 mM glycine, pH 2.5 and then neutralized by addition of 1 M Tris-HCl, pH 7.5. Protein samples were reduced with 5 mM dithiothreitol for 30 min at 37˚C and then alkylated with 15 mM iodoacetimide for 1 h at room temperature in the dark. Trypsin was added at a 1:50 protease/protein ratio and allowed to digest at 37˚C overnight or no longer than 16 h.

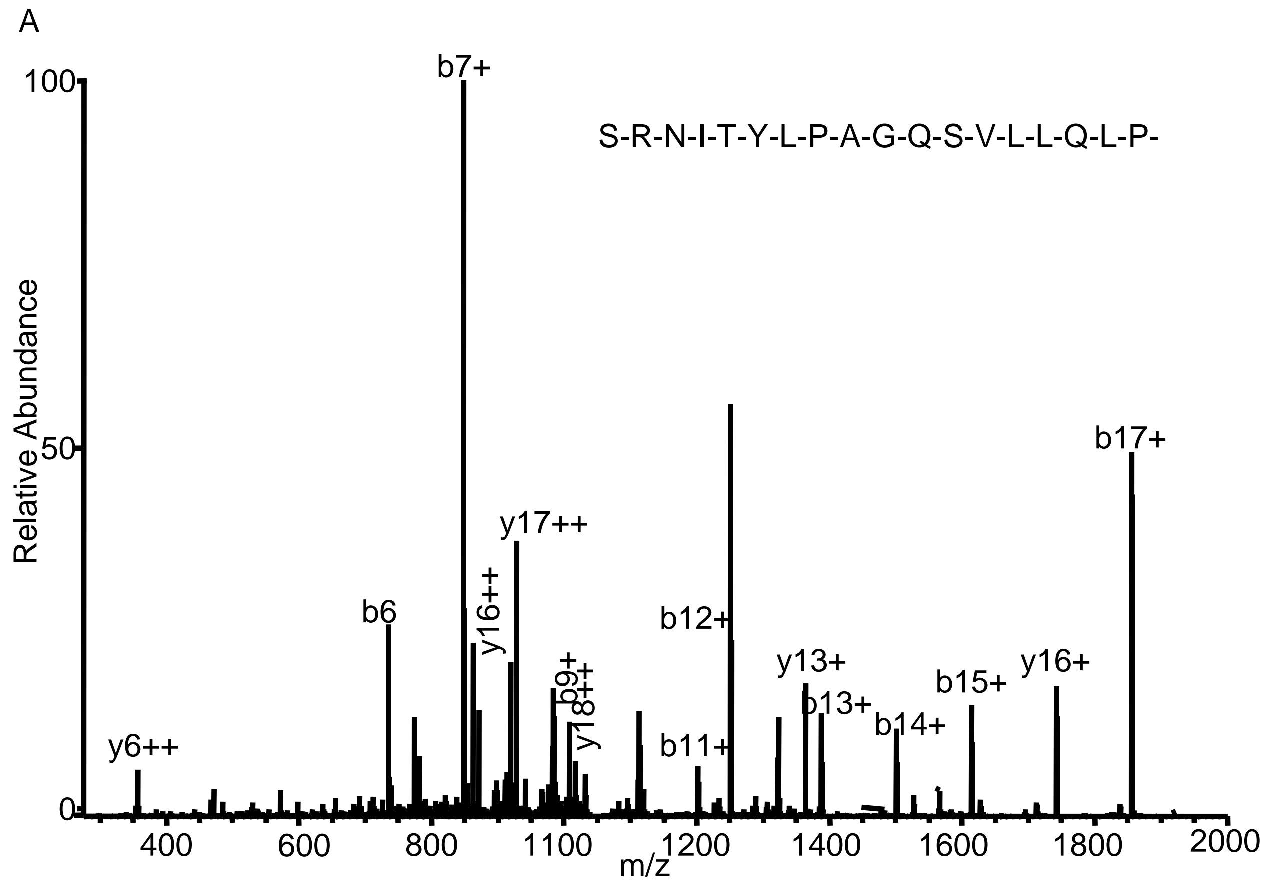
For the analysis of Syk-interacting proteins, 20 µl (50% slurry) of GFP nanotrap-Sepharose resin (Chromotek, Germany) was added to precleared lysates from cells expressing Syk-EGFP and incubated for 1 h with rotation at room temperature. GFP nanotrap beads were washed 3 times with lysis buffer and 3 times with H_2O. Proteins were eluted using 100 µl of 0.2 % RapiGest™ in 50 mM trimethylammonium bicarbonate (TMAB), pH 8.0, at 99˚C for 5 min. Proteins were reduced, alkylated and digested with trypsin as described above. RapiGest™ was removed by acidification with 50 mM HCl and incubation for 30 min at 37˚C.

NanoflowLC- MS/MS—Peptide samples were off-line loaded on a C18 trap column and introduced into an Agilent nanoflow 1100 HPLC system. The nanoflow LC capillary column with integrated electrospray emitter tip was constructed in-house as described (Ficarro, S. B. Zhang, Y. Lu Y., Moghimi, A.R., Askenazi, M., Hyatt, E., Smith, E.D., Boyer, L., Schlaeger, T.M., Luckey, C.J., and Marto, J.A. (2009) *Anal. Chem.* **81**, 3440-3447). The buffer used was 0.1% formic acid with the eluting buffer of 100% CH_3CN run over a shallow linear gradient over 60 min with a flow rate of 300 nl/min. The Agilent 1100 HPLC system was coupled online to a linear ion trap mass spectrometer (LTQ-Orbitrap XL or LTQ-XL; Thermo Fisher, San Jose, CA). The mass spectrometer was operated in the data-dependent mode in which a full scan MS was followed by MS/MS scans of the 10 most abundant ions with +2 to +3 charge states. The mass exclusion time was 180 s.

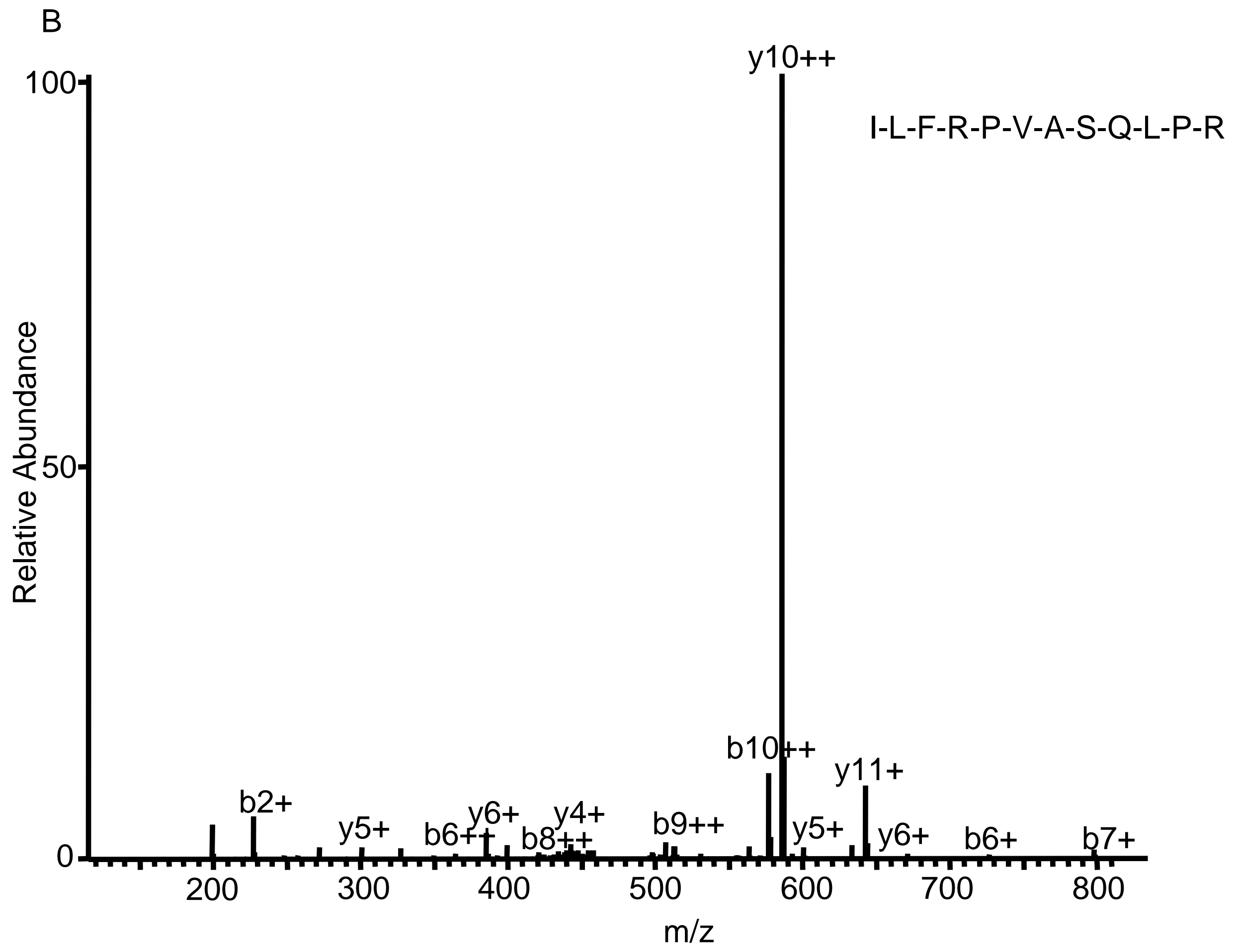
Peptide and protein identification—The MS/MS data were converted to a mzXML format using the open-source Trans-Peptide Pipeline (TPP) software (Version 2.9.4), and the resulting mzXML files were searched against *Gallus gallus* with *Mus musculus* Syk (32309 entries) or *Homo sapiens* from NCBI using the SEQUEST algorithm on the Sorcerer IDA server (Software Version 2.5.6; SageN, Inc, San Jose, CA, USA). Peptide mass tolerance was set at 10 ppm, and MS/MS tolerance was set internally by the software with accuracy values varying from 0 up to 1 amu. Search criteria included methionine oxidation (15.994 Da) and cysteine alkylation (57.021 Da). Searches were performed with semi-tryptic digestion and allowed maximum 2 missed cleavages on the peptides analyzed from the sequence database. Minimum protein and peptide probabilities were set to 0.8 and 0.9 respectively. For the analysis of phospho-Syk, search criteria included variable serine, threonine, and tyrosine phosphorylation (79.96 Da).



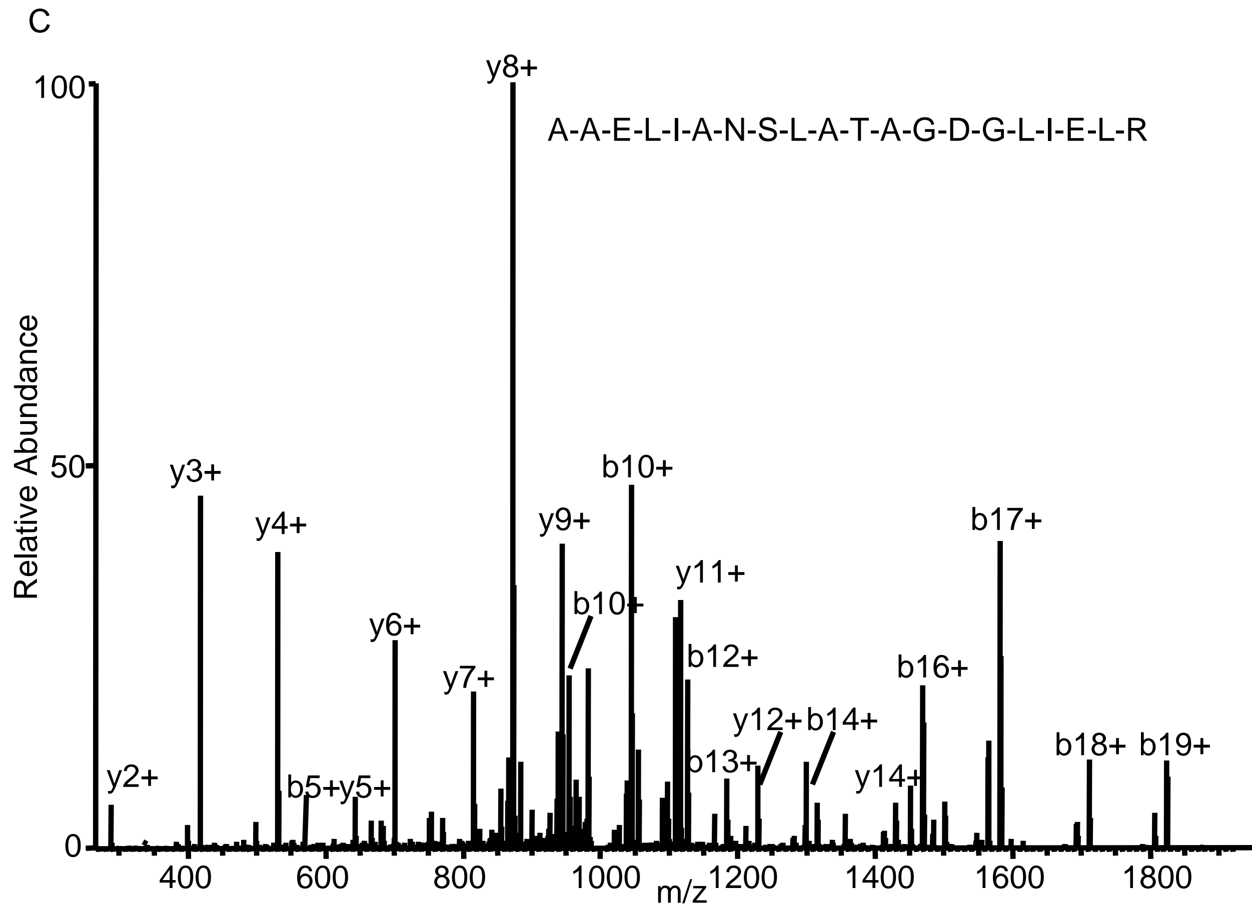
SUPPLEMENTAL FIGURE 1. **CID Spectra of Syk-derived phosphopeptide SYpSFPKPGHK, MH+ 1228.26.** The characteristic peptide bond fragment ions, type b and y ions, are labeled.



A
S-R-N-I-T-Y-L-P-A-G-Q-S-V-L-L-Q-L-P-
Relative Abundance
100
50
0
m/z
400
600
800
1100
1200
1400
1600
1800
2000
b7+
b17+
y17++
b6
y16++
b12+
b9+
y18++
y13+
b15+
y16+
b13+
b14+
y6++
b11+



B
y10++
I-L-F-R-P-V-A-S-Q-L-P-R
100
50
0
Relative Abundance
b2+
y5+
b6++
y6+
b8++
y4+
b9++
b10++
y5+
y11+
y6+
b6+
b7+
200
300
400
500
600
700
800
m/z



SUPPLEMENTAL FIGURE 2. **CID Spectra of PHB1-derived peptides isolated by phosphopeptide-affinity purification.** *A*, peptide SRNITYLPAGQSVLLQLPQ, MH+ 2099.419. *B*, peptide ILFRPVASQLPR, MH+ 1397.693. *C*, peptide AAELIANSLATAGDGLIELR, MH+ 1999.255. The characteristic peptide bond fragment ions, type b and y ions, are labeled.

SUPPLEMENTAL TABLE I

Identification of 14-3-3- and PHB1-derived peptides

Syk-EGFP was immunoprecipitated from stably transfected DT40 cells. Immune complexes analyzed for Syk-associated proteins by proteolysis followed by mass spectrometric analysis as described in Experimental Procedures.

Protein[a]	Peptide Probability	Precursor charge	Peptide sequence
14-3-3, alpha/beta	1	2	TAFDEAIAELDTLNEESYK
	1	2	DSTLIMQLLR
14-3-3, epsilon	1	3	AAFDDAIAELDTLSEESYK
	1	2	AASDIAM#TELPPTHPIR[b]
	1	2	VAGM#DVELTVEER[b]
14-3-3, zeta	0.99	2	YLAEVAAGDDKK
	1	2	GIVEQSQQAYQEAFEISK
PHB1	1	3	FDAGELITQR
	0.99	2	ILFRPVTAQLPR

[a]mzXML file was searched against NCBI chicken (*Gallus gallus*) protein database

[b]#-Methionine oxidation