Phospho-PepArray™ based Identification of Novel Protein Interaction Networks in Tyrosine Kinase Signaling Pathways

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Introduction

Protein phosphorylation mediates many critical cellular responses and is essential for many biological functions during development. About one-third of cellular proteins are phosphorylated, representing the phosphor-proteome, and phosphorylation can alter a protein's function, activity, localization and stability.

Tyrosine phosphorylation events mediated by aberrant activation of Receptor Tyrosine Kinase (RTK) pathways have been proven to be involved in the development of several diseases including cancer.

With the available phosphorylation data on various High throughput proteomic technology platforms, it is becoming increasingly evident that many proteins are interlinked with each other in multiple signaling pathways through multiple protein phosphorylation sites (pT, pS and pY). Hence it is becoming extremely hard to target a specific protein as a biomarker or for a drug.

Post –translational modifications (eg., pY) on proteins create multiple sub-groups of the same proteins which are pathway specific. Hence a sensible strategy is to target these phosphorylated sites that are pathway specific.

Here we present our microchip based (PepArray™) peptide microarray technology to characterize and identify novel protein interactions from cancer signaling pathways.

The interaction between phosphopeptides (PPEPs) and phosphoprotein binding domain containing proteins (PPBDs) on a microchip reveal novel protein cascades representing various signaling pathways through target binding.

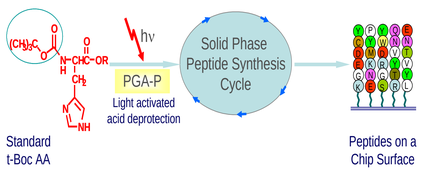
More than 10 families of PPBDs (SH2, PTB, WW, 14-3- etc) represent a vast majority of proteins in a signaling cascade. Proteins with phosphotyrosine modifications not only bind to proteins with SH2 domains but also activate downstream proteins (Kinases and phosphatases) to initiate a signaling cascade.

We illustrate Phosphotyrosine (pY) peptide interactions with an SH2 domain containing protein, GRB2 to identify protein complexes under various signaling cascades. We have used Breast cancer cells (T47D) and Human Umbilical vein cells (HUVEC) to demonstrate the differential signaling cascade mediated by differential protein-protein interaction networks.

Further focus will be on technology advancement through integration of high density peptide microarray with computational web tools to rapidly identify novel protein interaction networks in various cancers and neurodegenerative diseases and increase the sensitivity of detecting protein interactions.

µParaflo® Biochip Technology

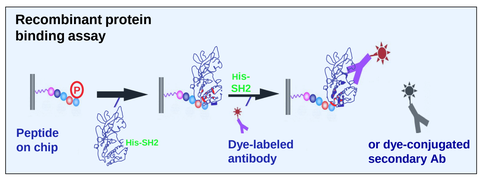
Thousands of different peptides can be synthesized *in situ* on 1 cm^2 microfluidic chip using standard t-Boc protecting amino acids and a photogenerated acid as deprotection reagent for the light-gated parallel synthesis (1,2).



Methods and Tools

Detection of PPEP mediated protein Binding

Recombinant SH2 protein and cell lysate binding assays



SH2 Proteins and cell lines Studied

SH2 proteins	#AA	MW	Function	Mobit[a]	Known Consensus sequence[b]	Tags/Dyes for dectection	Kd[c]	Ref
ZAP-70	619	70KD	Kinase	SH2 SH2 Y Kinase	[pY][DNS][EE][GA/V][L]	AntiZAP-70-Alexa 488	0.03-3 µM	1,2
Grb2	217	25KD	Adaptor	SH3 SH2 SH3	[pY] [IVQ/Y][N][LVY][F]	AntiGST-tyr lite 647 or AntiGST-Biotin+ Streptavidin-Alexa 594	0.01-1 µM	3,4
BTK	659	76KD	Kinase	PH TH SH3 SH2 Y Kinase	[pY][DEE][YTE]	Anti-His-(+) lite 647	2-20 µM	5,6
Src	536	60KD	Kinase	SH3 SH2 Y Kinase	[pY][EDT][EN/T][M/L]	Anti-His-(+) lite 555	0.04-07 µM	7,8

Cell lines	Description	Type	Protein for binding	&b and dyes
HUVEC	Human Umbilical Vein Endothelial Cells	Normal	Grb2	AntiGrb2+Antirabit-Alexa 647 conjugate
T47D	Human ductal breast epithelial tumor cell line	Cancer	Grb2	AntiGrb2+Antirabit-Alexa 647 conjugate

Note: a. the motifs of the SH2 proteins were from the website: http://sh2.uchicago.edu/
b. The consensus data were from "Liu et al. (2006) Mol Cell 22, 851-868".
c. the K_d values of the SH2 proteins were from the literatures listed in the Table.

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SH2-PPEP Microarray Design and Analysis

- **A SH2 domain interacting phosphopeptide (SH2-PPEP) microarray was designed.**
- **The PPEPs were from PepCyber ~PPEP (www.pepcyber.org).**
- **The PPEPs in the microarray are known substrates of SH2 domains**
- **Statistics of SH2-PPEP interactions**

Statistics of SH2 proteins and PPEPs in the microarray

Index	Number
SH2 proteins	93
PPEPs	994
PPEP proteins	432
Interactions	2455
Unique PPEPs	574
Unique PPEP proteins	161

Function	# of SH2 proteins	SH2 protein	# of ACC	# of PPEPs	# of PPEP protein	# of Unique PPEP	# of Unique PPEP proteins
Kinase	27	ABL1	NP_005146	59	21	8	4
		ABL2	NP_005149	29	5		
		BLK	NP_001706	11	6		
		BMX	NP_975010	2	4		
		BTK	NP_000052	1	1		
		CSK	NP_004374	16	13	4	2
		FER	NP_005237	3	2	2	1
		FES	NP_001996	27	23	13	7
		FGR	NP_005239	31	20		
		FRK	NP_002022	1	1		
		FYN	NP_002028	71	49	11	5
		HCK	NP_002101	6	4		
		ITK	NP_005537	9	4		
		JAK1	NP_002218	4	3		
		JAK2	NP_004963	7	4		
		JAK3	NP_000206	3	2		
		LCK	NP_005347	59	36	13	
		LYN	NP_002341	21	14	4	1
		MATK	NP_002369	10	8		
		PTK6	NP_005968	3	2	3	1
		SRC	NP_005408	62	51	17	7
		SYK	NP_003168	73	25	5	1
		TEC	NP_003208	6	2	3	
		TXK	NP_003319	2	4		
		TYK2	NP_003322	2	1		
		YES1	NP_005424	8	6		
		ZAP70	NP_001070	28	12	1	
Phophatases	2	PTPN11	NP_002825	220	109	58	14
		PTPN6	NP_002822	206	97	83	40
Ubiquitination	2	CBL	NP_005179	5	4	4	3
		CBLB	NP_733762	2	2		
Adaptor	9	CRK	NP_058431	75	21	19	1
		CRKL	NP_005198	99	27	15	2
		GRAP	NP_006604	4	3		
		GRAP2	NP_004801	9	6		
		GRB2	NP_002077	166	86	89	33
		NCK1	NP_006144	53	22	6	1
		NCK2	NP_003572	24	11	4	
		SLA	NP_006739	16	5		
		SLA2	NP_115590	7	3		
Phospholipid	7	INPPD	NP_005532	34	17		
		INPPL1	NP_001558	2	2	1	
		PIK3R1	NP_852664	166	76	83	17
		PIK3R2	NP_005018	30	14	1	1
		PIK3R3	NP_003620	21	5		
		PLCG1	NP_002651	105	42	12	2
		PLCG2	NP_002662	33	14	2	

Function	# of SH2 proteins	SH2 protein	# of ACC	# of PPEPs	# of PPEP protein	# of Unique PPEP	# of Unique PPEP proteins
Signal regulatoin	19	APS	NP_066189	6	3		
		CISH	NP_659508	27	7		
		DAPP1	NP_055210	3	2		
		GRB10	NP_005302	24	7	7	1
		GRB14	NP_004481	6	3	1	
		GRB7	NP_005301	21	11	1	1
		HSH2D	NP_116244	3	2		
		SH2B1	NP_056318	6	4	3	1
		SH2B3	NP_005466	4	3		
		SH2D1A	NP_002342	16	8	2	
		SH2D1B	NP_444512	12	5		
		SH2D2A	NP_003966	2	2		
		SH3BP2	NP_003014	17	14	6	2
		SHB	NP_003019	4	3		
Scaffold	5	SOCS1	NP_003736	9	5	1	
		SOCS2	NP_003868	12	6		
		SOCS3	NP_003946	15	10	1	
		SOCS4	NP_543143	2	2		
		SOCS5	NP_054720	2	2		
Transcription	7	BLNK	NP_037446	2	2	1	
		LCP2	NP_005556	3	1	3	
		MIST	NP_443196	16	4		
		SHC1	NP_892113	113	54	36	4
		SHC3	NP_058544	7	3		
		STAT1	NP_009330	6	18	1	3
		STAT2	NP_005410	5	4		
		STAT3	NP_644805	36	16	15	2
		STAT4	NP_003142	4	4	1	
		STAT5A	NP_003143	17	7	1	
		STAT5B	NP_036580	17	8		
		STAT6	NP_003144	7	4	2	
Small GTPase signaling	11	BCAR3	NP_003558	6	2		
		CHN1	NP_001813	4	3		
		CHN2	NP_004058	3	2		
		RASA1	NP_002881	55	18	7	1
		RIN1	NP_004283	4	2		
		RIN2	NP_061866	7	3		
		SH2D3A	NP_005481	4	3		
		SH2D3C	NP_005480	1	1		
		VAV1	NP_005419	40	22	11	5
		VAV2	NP_003362	14	6		
		VAV3	NP_006104	9	4		
Chromatin	4	TENC1	NP_056134	9	3		
		TNS1	NP_872174	1	1		
		TNS3	NP_073585	4	2		
		TNS4	NP_116254	2	2		

PPEP: phosphopeitde

PPEP protein: proteins containing PPEP

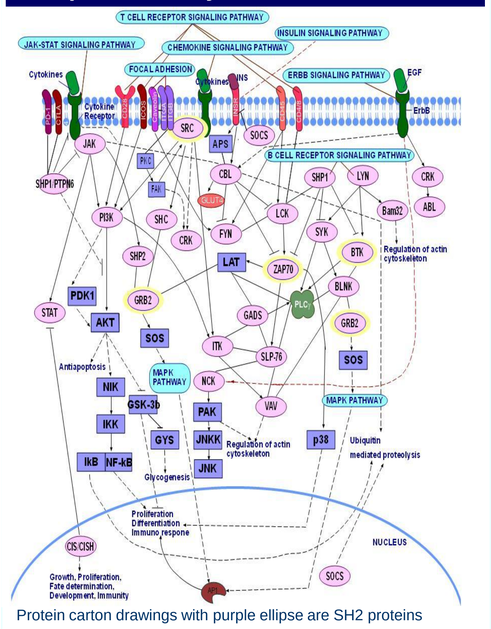
Unique PPEP: PPEP that uniquely binds to a SH2 domain

Unique PPEP protein: PPEP protein that uniquely binds to a SH2 domain

The SH2 domain proteins can be classified into 10 groups based on their functions.

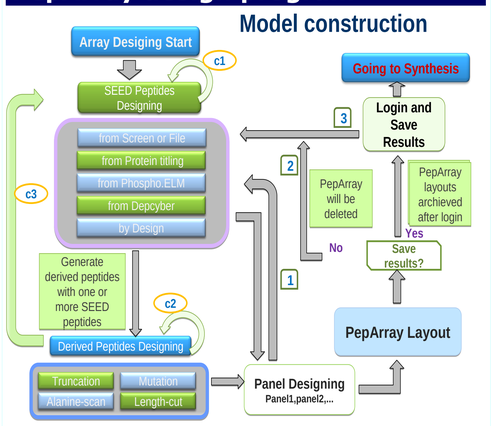
Reference: Liu, B.A, Jablonowski, K., Raina, M., Arcé, M., Pawson, T., Nash, P.D. (2006) The human and mouse complement of SH2 domain proteins-establishing the boundaries of phosphotyrosine signaling. Mol Cell. 22, 851-868.

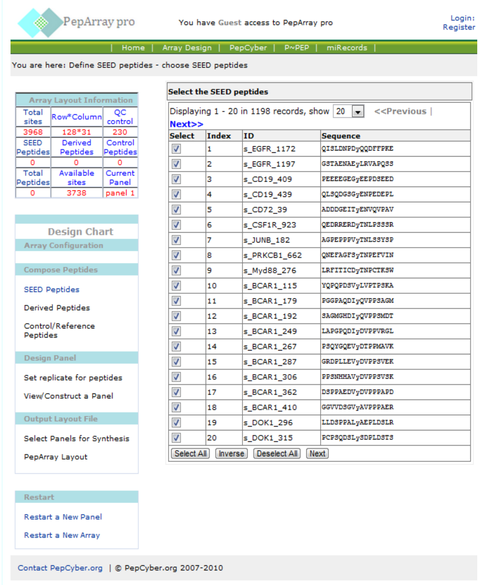
SH2 Protein Network and Cell Surface Receptor Pathways



Protein carton drawings with purple ellipse are SH2 proteins

PepArray pro – a user-driven PepArray design program



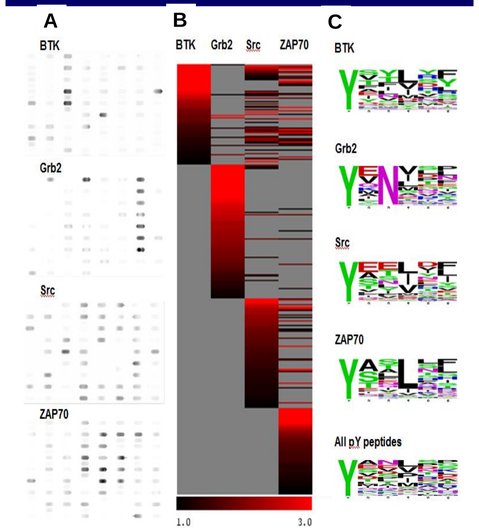


Select	Index	ID	Sequence
☑	1	s_EGFR_1172	QISLDNPDyQQDFFPKE
☑	2	s_EGFR_1197	GSTAENAEyLRVAPQSS
☑	3	s_CD19_409	PEEEEGEDyEEPDSEED
☑	4	s_CD19_439	QLSQDGSDyENPEDEPL
☑	5	s_CD72_39	ADDDGEITyENVQVPAV
☑	6	s_CSF1R_923	QEDRREADyINLSSSR
☑	7	s_JUNB_182	AGPEPPPVyINLSSYSP
☑	8	s_PRKCB1_662	QHEFAGFSyINPEFVIN
☑	9	s_Myd88_276	LRFITICDyPPCTKSW
☑	10	s_BCAR1_115	VQPQPDSYyLVTPSRA
☑	11	s_BCAR1_179	PGGPAQDIyQVPPSAGM
☑	12	s_BCAR1_192	SAGMGNDIyQVPPSMDT
☑	13	s_BCAR1_249	LAPGPQDIyDVPPVRGL
☑	14	s_BCAR1_267	PRQVPQEVyDTPPHAVK
☑	15	s_BCAR1_287	GRDPLLEVyDVPPSVEK
☑	16	s_BCAR1_306	PFSNRNAYyDVPPSVSK
☑	17	s_BCAR1_362	DSPPAEDYyDVPPPAPD
☑	18	s_BCAR1_410	GGVVDSGYyAVPPPAER
☑	19	s_DOK1_296	LLDSPPALyAEPLDSLR
☑	20	s_DOK1_315	PCPSQDSLySDPLDSTS

Link:
http://www.pepcyber.org/PepArray/

Results

Recombinant SH2 protein binding assay



Binding patterns and consensus sequences of BTK, Grb2, Src and ZAP70.

A. Binding profiles of 4 SH2 proteins on the same region of SH2-PPEP microarray.

B. Heatmap of binding intensities of 4 SH2 proteins to related PPEPs. The binding signals were normalized into Z values. The PPEPs with Z values ranked from 1.0-3.0 were selected to plot the heat map.

C. Consensus sequences of binding sequences of 4 SH2 proteins. The consensus sequences were generated by using Weblogo (http://weblogo.berkeley.edu/logo.cgi).

Results:

- By using four recombinant SH2 proteins, specific and distinct binding profiles were obtained.
- The consensus sequences are consistent with those reported in literatures.
- Recombinant binding assays can be used as "reference" to calibrate the cell lysate binding.

Signature Peptides/Panels of Four SH2 Proteins

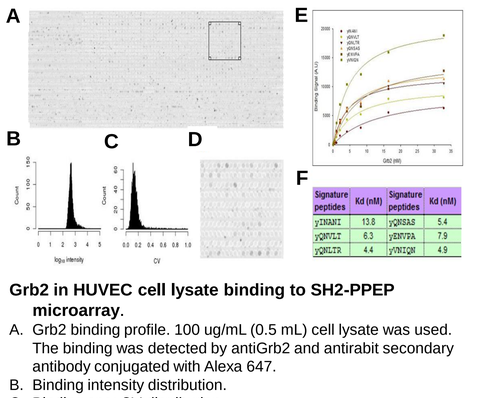
SH2 domain	PPEP_Seq	PPEP_Pro	Pho_site	Description
Grb2	yENVPA	LAX1	294	lymphocyte transmembrane adaptor 1
	yINANI	PTPN11	304	protein tyrosine phosphatase, non-receptor type 11
	yQNLTR	SPTAN1	2430	spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)
	yQNSAS	LAT2	193	linker for activation of T cells family, member 2
	yQNVLT	DST	633	dystonin
	yVNIQN	SHC1	313	SHC (Src homology 2 domain containing) transforming protein 1
BTK	yEYMNR	ERBB3	1260	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
	yIYVDP	PDGFRA	572	platelet-derived growth factor receptor, alpha polypeptide
	yLYLVV	EPOR	454	erythropoietin receptor
	yRFLYH	PTPN18	281	protein tyrosine phosphatase, non-receptor type 18 (brain-derived)
	yTDPSY	WASF3	151	WAS protein family, member 3
	yTFLMG	SSTR1	226	somatostatin receptor 1
	yVFFPS	IFNAR1	466	interferon (alpha, beta and omega) receptor 1
Src	ySYQDI	CBL	868	Cas-Br-M (murine) ecotropic retroviral transforming sequence
	yEEMYP	CCNB1	257	cyclin B1
	yEYMDV	ERBB3	1221	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
	yVIVEY	FGFR2	555	fibroblast growth factor receptor 2
	yVLPI	KDR	1174	kinase insert domain receptor (a type III receptor tyrosine kinase)
	yVLSF	PDGFRA	719	platelet-derived growth factor receptor, alpha polypeptide
	yAEID	PTK2	418	PTK2 protein tyrosine kinase 2
	yLIYVF	STAT5B	664	signal transducer and activator of transcription 5B
ZAP70	yASLNF	CD33	340	CD33 molecule
	ySHLGG	CD3D	160	CD3d molecule, delta (CD3-TCR complex)
	ySLLMH	FCGR2B	292	Fc fragment of IgG, low affinity IIb, receptor (CD32)
	ySALTC	IL4R	713	interleukin 4 receptor
	yTALQR	NFAM1	220	NFAT activating protein with ITAM motif 1
	yQFLVD	PTPRC	1216	protein tyrosine phosphatase, receptor type, C

The signature peptides/panels were identified by two steps:

(1) Based on *in silico* analysis, unique PPEPs of a SH2 protein or a unique PPEPs combination (panel) were screened.

(2) Based on binding assays, the unique peptides/panel of a SH2 protein need to show high affinity binding and without overlapping among the four SH2 proteins.

Grb2 in Cell lysate binding assay



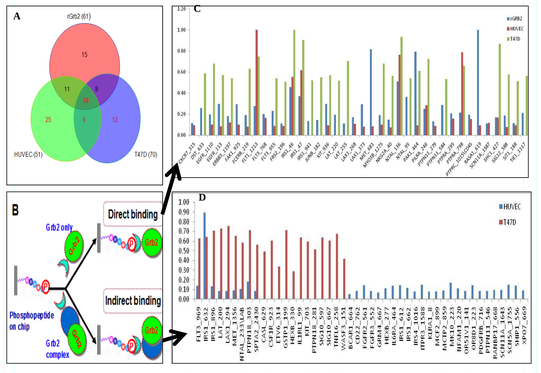
Grb2 in HUVEC cell lysate binding to SH2-PPEP microarray.

A. Grb2 binding profile. 100 ug/mL (0.5 mL) cell lysate was used. The binding was detected by antiGrb2 and antirabit secondary antibody conjugated with Alexa 647.
B. Binding intensity distribution.
C. Binding spot CV distribution.
D. Close-up image of the small region boxed in panel A.
E. Grb2 titration in the HUVEC cell lysate. The titration curves for 6 signature PPEPs were plotted. It was shown that 0.5 nM Grb2 can be probed.
F. The Kd values were calculated for 6 signature peptides of Grb2.

Major points:

- The cell lysate binding to SH2-PPEP microarray was uniform and consistent.
- The signature peptides of Grb2 might be used for detecting the Grb2 in cell lysates with sensitivity of low nM level.

Direct and Indirect Grb2 mediated binding of PPEPs



A. Venn diagram for three datasets of rGrb2 (recombinant Grb2), Grb2 in HUVEC and in T47D.

B. Endogenous Grb2 in cell lysate can bind to PPEPs in two patterns: direct binding and indirect binding.

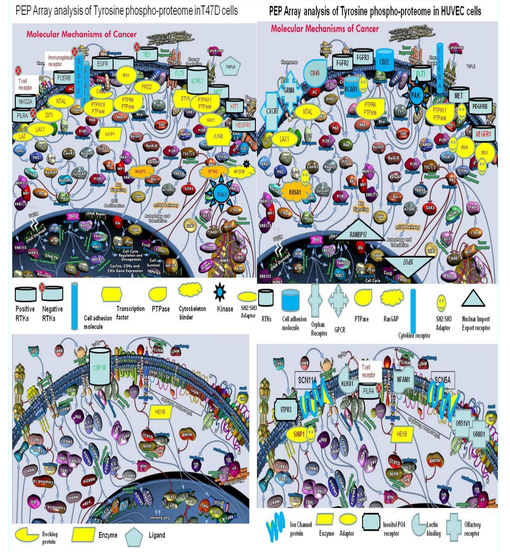
C. Grb2 in cell lysates can bind to the same PPEPs as rGrb2 does, indicating that Grb2 itself directly binds to these PPEPs—**direct binding**.

D. Grb2 in cell lysates can bind to the PPEPs that rGrb2 can not, indicating that Grb2 indirectly binds to these PPEPs via other SH2 domain proteins in the Grb2 complex—**indirect binding**

Results:

- Differential binding patterns were observed in T47D and HUVEC cell binding assays.
- HUVEC and T47D have different binding profile, which can be used for identifying different binders of Grb2 in two cell lines.

Grb2 mediated pY proteome networks in HUVEC and T47D cells



Summary

A strategy has been outlined for identifying signature peptide probes that could serve as signaling pathway specific markers to distingush normal and diseased protein samples (e.g., Identification of cancer types and subtypes).

By combining *in silico* analysis (PepArray pro) and binding experiments, we screened signature peptides/panels for Grb2, BTK, Src and ZAP70. With signature peptides of Grb2, we could detect Grb2 in cell lysates with sensitivity of low nM level.

We demonstrate a method to investigate SH2 protein interactions and related pathways in cells. From *in silico* analysis and recombinant protein binding assay to cell lysate binding assay, we found differential binding patterns of GRB2 protein in cell lysates (direct &indirect binding), which lead us to identify Grb2 mediated SH2 protein networks and related pathways typical to HUVEC and T47D cells.

The future direction is to develop this technology for Biomarker screening in patient sample, focusing on personalized medicine to help physicians to diagnose, stratify and select treatment options for diseases.

Acknowledgements

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Contact Information

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