Src Homology 2 Domain-based High Throughput Assays for Profiling Downstream Molecules in Receptor Tyrosine Kinase Pathways*

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Src homology 2 (SH2) domains are evolutionary conserved small protein modules that bind specifically to tyrosine-phosphorylated peptides. More than 100 SH2 domains have been identified in proteins encoded by the human genome. The binding specificity of these domains plays a critical role in signaling within the cell, mediating the relocalization and interaction of proteins in response to changes in tyrosine phosphorylation states. Here we developed an SH2 domain profiling method based on a multiplexed fluorescent microsphere assay in which various SH2 domains are used to probe the global state of tyrosine phosphorylation within a cell and to screen synthetic peptides that specifically bind to each SH2 domain. The multiplexed, fluorescent microsphere-based assay is a recently developed technology that can potentially detect a wide variety of interactions between biological molecules. We constructed 25-plex SH2 domain-GST fusion protein-conjugated fluorescent microsphere sets to investigate phosphorylation-mediated cell signaling through the specific binding of SH2 domains to activated target proteins. The response of HeLa, COS-1, A431, and 293 cells and four breast cancer cell lines to epidermal growth factor and insulin were quantitatively profiled using this novel microsphere-based, multiplexed, high throughput assay system. ***Molecular & Cellular Proteomics 5:959–968, 2006.***

Phosphorylation, as one of the predominant events in cellular signaling, plays a crucial role in the propagation of cellular information that is critical to biological processes, including cell proliferation, survival, migration, differentiation, and gene transcription. Tyrosine phosphorylation is mediated by protein-tyrosine kinases (PTKs),[1] and more than 90 PTK genes have been identified in the human genome (1). One of the most important groups of PTKs is the receptor tyrosine kinases (RTKs). Among the 90 PTKs identified, 58 are RTKs (1, 2). These kinases are expressed on the cell surface and act as biosensors to initiate a series of cellular responses to the presence of growth factors and hormones in the cellular microenvironment. RTKs are activated by the binding of specific ligands, which leads to the dimerization of the receptor and autophosphorylation of specific receptor tyrosine residues. These phosphotyrosine residues deliver the signals to downstream molecules via their interactions with proteins containing the Src homology 2 (SH2) domain (3, 4–6). SH2 domains are ~100 amino acids long and recognize phosphotyrosine residues within specific sequence contexts. They are found in proteins with diverse biochemical functions, including growth factor receptors and adaptor proteins. A total of 115 SH2 domains have been identified in the human genome. Through these domains, receptors and downstream signaling molecules are connected, allowing signals to be transmitted within a cell. Deregulation of this tightly controlled system by the overexpression, amplification, and mutation of components in the signaling network is frequently linked to human cancer (7–13).

Epidermal growth factor receptor (EGFR) is one of the most studied RTKs. Recent evidence has implicated EGFR in human breast cancer development (1, 8, 13). This evidence includes the functional role of EGFR as a proto-oncogene in viruses, the pathophysiological effect of EGFR mutants, its overexpression in several cancer types, and the inverse correlation of its overexpression with estrogen receptor status (14, 15). There are four known members in the EGFR family: EGFR/HER1, HER2, HER3, and HER4, which are activated by different stimuli. The members of the EGFR family form several possible homo- and heterodimeric receptor complexes, and the ratio of homo- to heterodimeric complexes may be influenced by the expression pattern of these EGFR family members within the cell (16). These receptor complexes activate multiple downstream signal transduction pathways by the recruitment of SH2 domain-containing proteins such as Cbl, Src, Shc, Shp2 (also known as PTPN11), Nck, Vav, and Crk. Activation of these SH2 proteins subsequently leads to signal transduction through various signaling pathways including the Ras-mitogen-activated protein kinase,

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Received, January 17, 2006, and in revised form, February 2, 2006
Published, MCP Papers in Press, February 13, 2006, DOI 10.1074/mcp.T600002-MCP200

[1] The abbreviations used are: PTK, protein-tyrosine kinase; SH2, Src homology 2; SH3, Src homology 3; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; RTK, receptor tyrosine kinase; MFI, median fluorescence intensity; cont, untreated control.

Nck-c-Jun N-terminal kinase, phospholipase Cγ-protein kinase C, and phosphoinositide 3-kinase-Akt pathways (16). The key to understanding how RTKs modulate downstream signaling pathways is identifying which SH2 domain-containing proteins are recruited to bind to the active tyrosine phosphoproteins.

A number of techniques have been used to study the interactions of proteins with phosphoproteins (2, 17). Co-immunoprecipitation of tyrosine-phosphorylated proteins complexed with their interacting partners followed by identification of the proteins by immunoblotting is a commonly used method of analysis. The antibody used for isolation of the complex can either be specific to the phosphorylated tyrosine residue or to the tyrosine kinase protein. Because this assay characterizes one target at a time, this method would be labor-intensive and time-consuming if it were used to characterize which particular SH2 domain, among the 115 possible SH2 domains, binds to EGFRs. However, when the antibodies are used in conjunction with MS detection systems, tyrosine-phosphorylated proteins can be successfully detected at low levels, making it a powerful tool for phosphotyrosine profiling. The large scale application of the MS approach is limited by the high cost of instrument set-up and maintenance. Therefore, although this method is well suited to the initial characterization of phosphotyrosine proteins, it is not ideal for routine use in profiling applications (for a review, see Ref. 2).

Gene chip technology is recognized as one of the most powerful methods for profiling gene expression in a given sample, especially in its application to identifying genes implicated in human disease (18, 19). However, measurements of mRNA transcript levels do not always accurately represent the corresponding protein abundance or activity, due in part to the post-translation modification of proteins. This may explain the increasing demand for new technologies to analyze the functions of proteins, such as tyrosine phosphorylation and protein interactions mediated by this process. Antibody arrays represent one of the earliest forms of protein arrays and have been used to profile the phosphorylation of tyrosine residues (20). Detection of phosphoproteins by antibody arrays often requires two antibodies targeting the same protein: those raised against specific target proteins for capture and those raised against phosphotyrosine residues for detection. Antibody arrays are useful in profiling the phosphotyrosine status of proteins but do not provide information on downstream signaling proteins that bind to phosphotyrosine proteins.

Here we describe a SH2 domain-based assay we developed to profile the interaction of RTKs or other phosphotyrosine proteins with downstream molecules in their signaling pathways. This assay is a microsphere-based method in which various SH2 domains are coupled to different microspheres to capture any binding proteins in a sample. The SH2 domain-bound proteins are then detected with specific antibodies against either phosphotyrosine residues or specific phosphotyrosine proteins. We present results indicating how this methodology can determine the interactions of specific SH2 domains with RTKs.

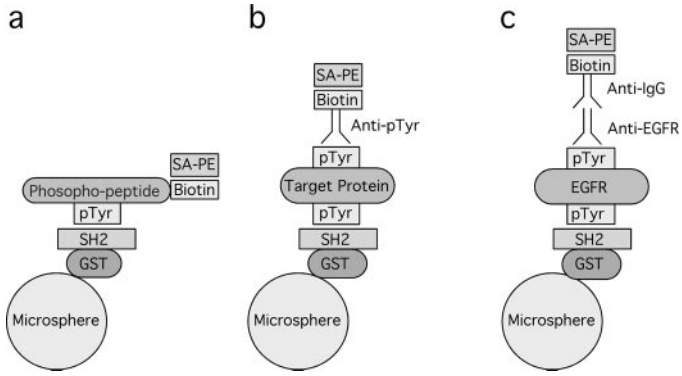

FIG. 1. **Scheme of the SH2 domain-conjugated fluorescent microsphere-based assay.** Different GST-SH2 domain fusion proteins are conjugated to each microsphere region. Biotinylated phosphopeptides can be screened directly (*a*). Specific binding of phosphotyrosine proteins to particular SH2 domains are detected with anti-phosphotyrosine (*pTyr*) (*b*) or anti-RTK such as anti-EGFR (*c*). *SA-PE*, streptavidin-phycoerythrin.

TABLE I
Peptide probes showed in this study

The phosphotyrosine residues are underlined.

Peptide ID	Target domain	Peptide sequence	Protein name
L0000227	PDZ	PPSEKHFRETEV	Tax protein from human T-lymphotropic virus 1
L0011753	SH3	PIPPPLPLLPPCGY	WUGSC:H_DJ0871B15.2 protein
L0007750	SH2	LASKYEEIYPPE	Cyclin A1
L0015512	SH2	DPNGYMMMSPSG	Insulin receptor substrate-1 (IRS-1)
L0015629	SH2	HEDLYIIPIPNC	Insulin-like growth factor-binding protein 4 precursor (IGFBP-4)
L0015782	SH2	HRELYLRVELSD	Similar to *Mus musculus* partial B-IND1 protein
L0015849	SH2	LIGFYQPDEPLT	GDP-mannose pyrophosphorylase A
L0015990	SH2	FPYYYANLGLKPG	Putative tumor suppressor protein EXT1
L0016204	SH2	NRTGYANVTIYK	Interleukin 1 receptor-like 1 precursor (ST2 protein)
L0016220	SH2	TAGYYPNPPLVL	DJ486I3.1 (novel protein)
L0016305	SH2	DYQVYLNASKVPG	KIAA1436 protein

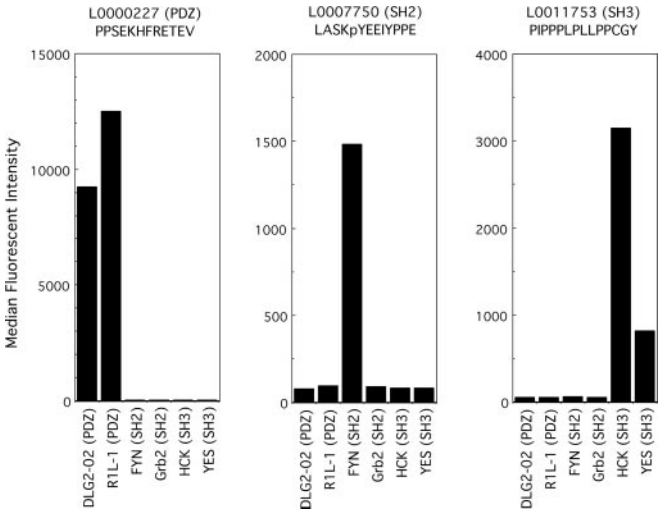

FIG. 2. **Specificity of the SH2, SH3, or PDZ domains conjugated on microspheres.** The three peptides (L0000277, L0007750, and L0011753) were separately incubated with the mixture of six domain-conjugated microspheres and labeled with streptavidin-phycoerythrin. The formation of the protein-peptide-phycoerythrin complex was analyzed on the Luminex 100.

EXPERIMENTAL PROCEDURES

GST Fusion Proteins—Gene sequences encoding various SH2, SH3, and PDZ domains were extracted from ProDom (protein.toulouse.inra.fr/prodom/current/html/form.php), SMART (smart.embl-heidelberg.de/), PROSITE (www.expasy.org/prosite/), and InterPro (www.ebi.ac.uk/interpro/) databases. The coding regions for each domain were amplified by PCR from a human cDNA library (Panomics) and cloned into pGEX-4T1 plasmid expression vectors (Amersham Biosciences). The identities of the resulting vectors were confirmed by sequencing, and they were then used to transform *Escherichia coli* DH5α cells. After induction of protein expression with 0.1 mM isopropyl 1-thio-D-galactopyranoside (Sigma) for 2–4 h, the bacteria were resuspended in a lysis buffer and disrupted by sonication. Following centrifugation at 10,000 $\times$ *g* for 20 min, the induced proteins were adsorbed to bead-immobilized glutathione (Clontech). Soluble GST fusion proteins were obtained by elution with 2 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. Purified fusion proteins were dialyzed twice against 1 liter of PBS at 4 °C for at least 5 h.

Cell Lines and Cell Culture—Human HeLa, human embryonal kidney cell line 293, human A431, human COS-1, human Hcc1806, human MCF7, human Hcc1599, and human Hcc1143 cell lines were obtained from the American Type Culture Collection (ATCC). Cells were maintained in minimum Eagle's medium containing 10% fetal bovine serum at 37 °C in a humid atmosphere of 5% CO_2. Minimum Eagle's medium and fetal bovine serum were purchased from ATCC.

Multiplexed Fluorescent Microsphere Assays—200 μl of COOH fluorescent microspheres (Luminex) were activated by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and sulfo-*N*-hydroxysuccinimide (Pierce) according to the manufacturer's instructions and conjugated with 50 μg of PDZ-GST, SH3-GST, or SH2-GST fusion proteins. GST not fused with any domains was also conjugated onto the microsphere for negative control to confirm no cross-reactions between GST and synthetic peptides or cell lysates. The GST fusion protein-conjugated microspheres were diluted in PBS with 1% BSA to 200 microspheres/μl for each region.

For peptide binding assays, 25 μl of the diluted microsphere mixture was incubated with 25 μl of biotinylated peptide (Panomics) at various concentrations in PBS with 1% BSA at room temperature for 30 min, then mixed with 25 μl of 10 μg/ml streptavidin-phycoerythrin (Invitrogen) in PBS with 1% BSA, and incubated for 30 min at room temperature. 50 μl of the mixture was analyzed on a Luminex 100 instrument (Luminex). Detection conditions were set as follows: flow rate, fast; events/bead, 100; sample volume, 50 μl.

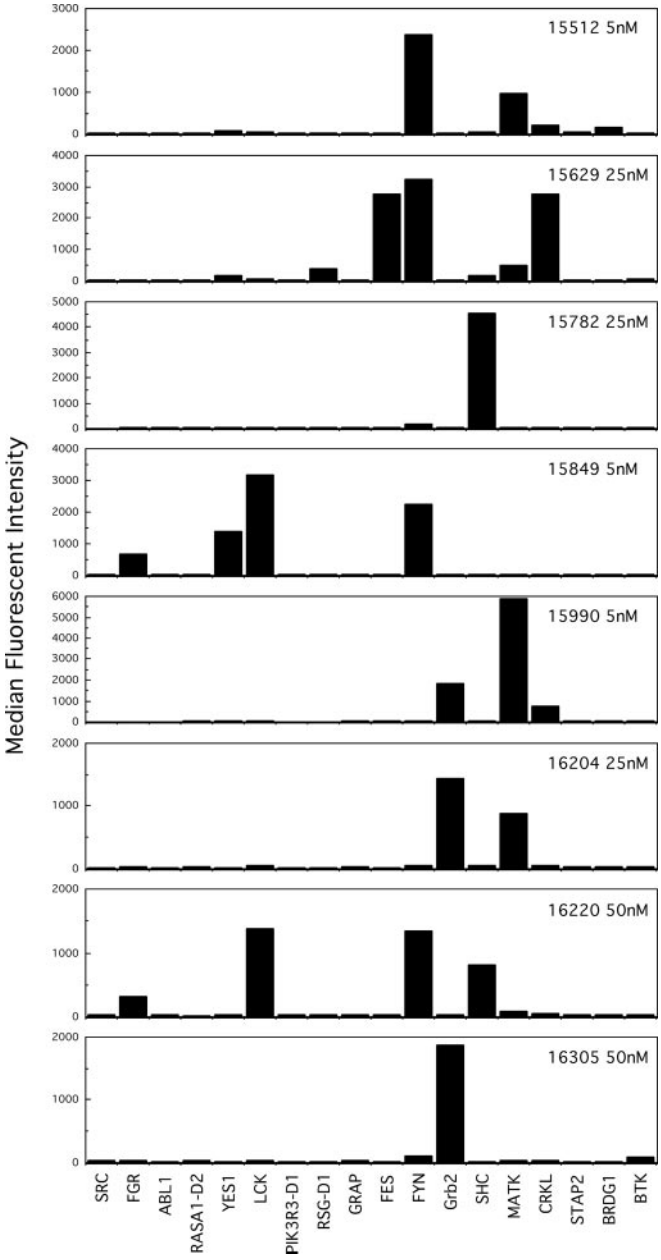

FIG. 3. **Profiling specific interactions between SH2 domains and phosphopeptides.** Eight phosphotyrosine-containing synthetic peptides were screened using the 18-plex SH2 domain profiling system. Each phosphopeptide, at the indicated concentration, was incubated with 18-plex SH2-conjugated microspheres, and the binding of the peptide to the beads was analyzed on the Luminex 100. Specific binding was observed at concentrations of peptide as low as 5 nM.

For assays of cell lysates, 75 μl of cell lysates, which contains 4 μg/μl proteins, were mixed with 25 μl of the 25-plex SH2 microsphere mixture (5000 microspheres in 25 μl) in PBS with 1% BSA in a well of a manifold filter plate (Millipore). The mixture was incubated for 1 h at

TABLE II
Summary of the 25-plex SH2-microsphere designs

Gene symbol	Gene name	NCBI accession no.	Bead region
PIK3R1-D1	Phosphoinositide 3-kinase, regulatory subunit 1 (p85 α)	NM_181504	10
PIK3R1-D2	Phosphoinositide 3-kinase, regulatory subunit 1 (p85 α)	NM_181504	11
CSK	c-*src* tyrosine kinase	NM_004383	12
SRC	v-*src* sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	NM_005417	21
FGR	Gardner-Rasheed feline sarcoma viral (v-*fgr*) oncogene homolog	NM_005248	22
ABL1	v-*abl* Abelson murine leukemia viral oncogene homolog 1	NM_007313	23
RASA1-D1	RAS p21 protein activator (GTPase-activating protein) 1	NM_022650	28
RASA1-D2	RAS p21 protein activator (GTPase-activating protein) 1	NM_022650	24
YES1	v-*yes-1* Yamaguchi sarcoma viral oncogene homolog 1	NM_005433	25
LCK	Lymphocyte-specific protein-tyrosine kinase	NM_005356	26
PIK3R3-D1	Phosphoinositide 3-kinase, regulatory subunit 3 (p55 γ)	NM_003629	27
GRAP	GRB2-related adaptor protein	NM_006613	29
FES	Feline sarcoma oncogene	NM_002005	30
PIK3R2-D1	Phosphoinositide 3-kinase, regulatory subunit 2 (p85 β)	NM_005027	81
PIK3R2-D2	Phosphoinositide 3-kinase, regulatory subunit 2 (p85 β)	NM_005027	82
FYN	FYN oncogene related to SRC, FGR, YES	NM_002037	83
Grb2	Growth factor receptor-bound protein 2	NM_025199	84
PTPN11-D1	Protein-tyrosine phosphatase, non-receptor type 11 (Noonan syndrome 1)	NM_018508	85
PTPN11-D2	Protein-tyrosine phosphatase, non-receptor type 11 (Noonan syndrome 1)	NM_018508	86
SHC1	SHC (*Src* homology 2 domain-containing)-transforming protein 1	NM_183001	87
MATK	Megakaryocyte-associated tyrosine kinase	NM_139355	95
CRKL	v-*crk* sarcoma virus CT10 oncogene homolog (avian)-like	NM_005207	96
STAP2	Signal-transducing adaptor protein-2	NM_017720	97
BRDG1	BCR downstream signaling 1 (BRDG1)	NM_012108	98
BTK	Bruton agammaglobulinemia tyrosine kinase	NM_000061	99

room temperature with agitation, avoiding exposure to light. After incubation, the plate was washed three times with 200 μl of PBS with 1% BSA. For Tyr(P) detection, 100 μl of biotinylated anti-Tyr(P) antibody RC20 (BD Pharmingen) in PBS with 1% BSA was added to each well and incubated for 1 h at room temperature with agitation, avoiding exposure to light. For EGFR detection, an anti-active EGFR antibody (BD Pharmingen) was used. The plate was washed as above, and streptavidin-phycoerythrin was added. For EGFR detection, an incubation step with a biotinylated secondary antibody was conducted prior to the streptavidin-phycoerythrin incubation. The fluorescent labeled mixture was then washed twice and resuspended with 75 μl of PBS with 1% BSA. A 50-μl sample of each well was analyzed on the Luminex 100. Detection conditions were set as follows: flow rate, fast; events/bead, 100; sample volume, 50 μl.

RESULTS

Design of Microsphere-based SH2 Domain Binding Assay—As diagramed in Fig. 1, various SH2 domains are first conjugated onto different microspheres (21). A biotinylated phosphopeptide or cell lysate is then incubated with the beads. The bound biotinylated phosphopeptides are detected using a streptavidin-labeled fluorescent dye (Fig. 1*a*), whereas the bound phosphotyrosine proteins from the cell lysate are detected using an antibody specific to either phosphotyrosine residues or to a specific protein with subsequent addition of biotinylated secondary antibody and streptavidin-labeled fluorescent dye (Fig. 1, *b* and *c*). Detection of the streptavidin-labeled fluorescent dye indicates whether binding to the SH2 domains is occurring, and the microsphere-encoded fluorescent signals identify the particular microsphere regions to which each SH2 domain is conjugated. Therefore, the binding of a specific phosphotyrosine protein target to an SH2 domain can be profiled immediately. Furthermore because the assay is in a 96-well plate-based format, multiple samples can be analyzed simultaneously.

Specificity and Sensitivity of the SH2-conjugated Microsphere Assays—We amplified all 115 identified human SH2 domains from a pool of human cDNAs and cloned the amplified cDNAs into the pGEX-4T1 expression vector. For proteins that contain two SH2 domains, we cloned each SH2 domain separately and labeled the N- and C-terminal domains D1 and D2, respectively (*e.g.* PIK3R1-D1 and PIK3R1-D2 indicate N-terminal and C-terminal SH2 domains of PIK3R1, respectively). The identities of these clones were confirmed by sequencing analysis. The SH2 domains were expressed as GST fusion proteins, which were purified using glutathione-Sephadex columns, and the purified proteins were examined by SDS gel electrophoresis. To determine the specificities of the interactions between the phosphotyrosine peptides and the SH2 domains, we conjugated two different GST-SH2 domain fusion proteins (GST-FYN and GST-Grb2) onto regions 83 and 84, respectively, of the microspheres (Luminex Multi-Analyte COOH Microspheres). In addition, we conjugated two PDZ domain fusions (GST-DLG2 and GST-RIL-1) onto microsphere regions 81 and 82 and two SH3 domain fusions (GST-HCK and GST-YES) onto regions 85 and 86, respec-

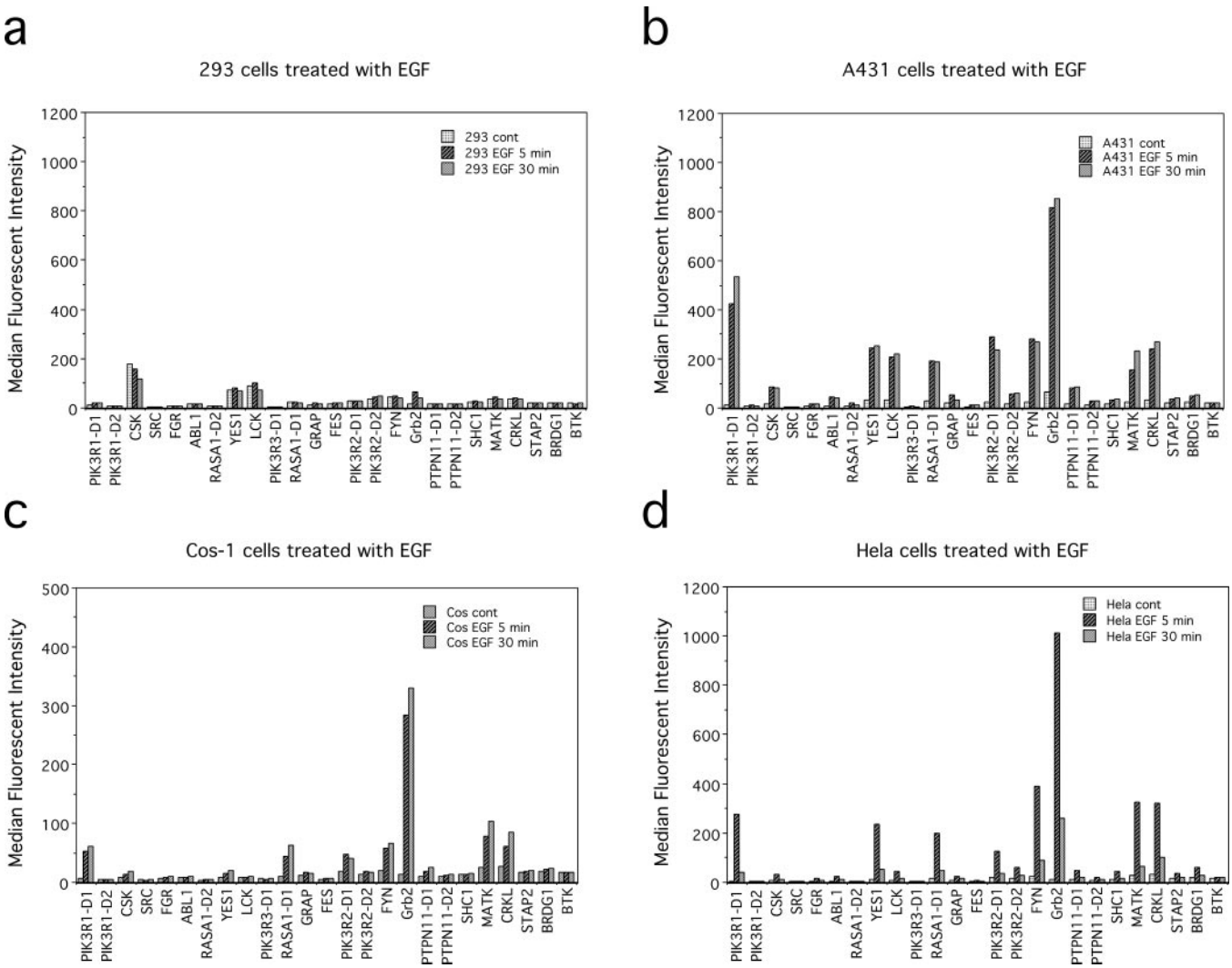

FIG. 4. **Profiling of tyrosine phosphoprotein-SH2 interactions in EGF-treated cells detected by anti-Tyr(P) antibody.** The four cell lines indicated were treated with EGF for 5 or 30 min prior to cell lysis, and then the cell lysates were assayed with 25-plex SH2 domain microsphere sets. Background signals for each region obtained from the assay with no cell lysates were less than 50 MFI. *a*, 293 cells. *b*, A431 cells. *c*, COS-1 cells. *d*, HeLa cells.

tively. Successful protein conjugation was confirmed using biotinylated anti-GST antibodies (data not shown). Three biotinylated polypeptides, L0000277, L0007750, and L0011753 that specifically bind the PDZ, SH2, and SH3 domains, respectively (Table I), were used to test whether the conjugated proteins retain their binding activity. The bound peptides were detected using fluorescent dye-conjugated streptavidin. As shown in Fig. 2, L0007750 specifically bound to the GST-FYN-conjugated microsphere region, L0011753 bound strongly to GST-HCK and weakly to GST-YES, and L0000277 bound to both GST-DLG2 and GST-RIL-1. No cross-reaction of the peptides with the other families of domains were found, demonstrating that the conjugated GST-domain fusion proteins retained their specific binding activity.

To examine the binding specificity of the conjugated SH2 domains toward phosphopeptides, we constructed 18-plex GST-SH2 microsphere sets and used them to screen for the binding of 106 biotinylated, synthetic phosphotyrosine peptides that correspond to phosphotyrosine sites within various proteins (Supplemental Table 1). Each of the 106 peptides was added to individual wells among 96-well plates, and several sets of these 96-well plates were then made with peptide concentrations ranging from 5 to 100 nM. The 18-plex GST-SH2 microspheres were allowed to incubate with the peptides in each well, and binding of these peptides to the SH2 domains on the microsphere was detected using fluorescent dye-conjugated streptavidin. We observed a wide range of binding specificities of the SH2-GST microspheres for the phosphopeptides. The binding patterns of eight peptides to the SH2 domains, for instance, are shown in Fig. 3. Although these phosphopeptides exhibited large differences in

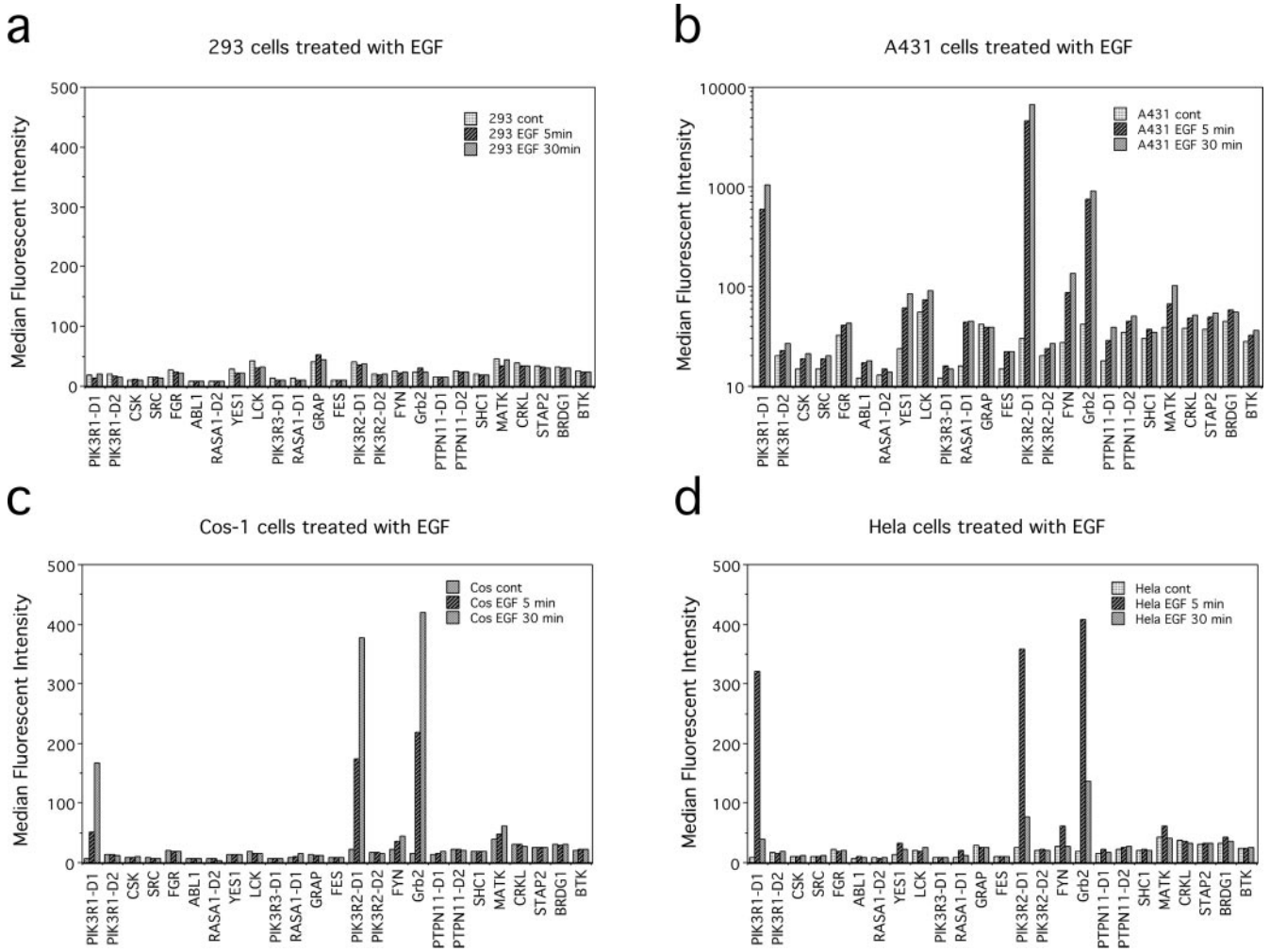

FIG. 5. **Profiling of EGFR-SH2 interactions in EGF-treated cells detected by anti-active EGFR antibody.** Each cell lysate used for this figure was the same as those in Fig. 4. The scale on the *y axis* for *b* is logarithmic to accommodate the significantly high signal value for PIK3R2-D1. Background signals for each region obtained from the assay with no cell lysates were less than 50 MFI. *a*, 293 cells. *b*, A431 cells. *c*, COS-1 cells. *d*, HeLa cells.

their binding specificities for the SH2 domains, the overall binding affinity was relatively high, and in most cases specific binding was detected at peptide concentrations as low as 5 nM.

Assay for Profiling the EGF-induced Activation of Phosphoproteins—To profile the binding of phosphotyrosine proteins in a cell lysate to SH2 domains, we conjugated 25 SH2-GST fusion proteins onto different regions of the microspheres as shown in Table II. For this analysis, we prepared cell lysates from the commonly used 293, A431, COS-1, and HeLa cell lines. These cells were treated with 100 ng/ml EGF for 5 or 30 min prior to cell lysis. To profile the binding of phosphotyrosine proteins to SH2 domains, we incubated the cell lysates with 25-plex microspheres followed by detection of the bound phosphotyrosine proteins with antibodies. Because most phosphotyrosine proteins, including RTKs, contain multiple phosphorylation sites, they can be detected by anti-phosphotyrosine antibodies while simultaneously bound to SH2 domains.

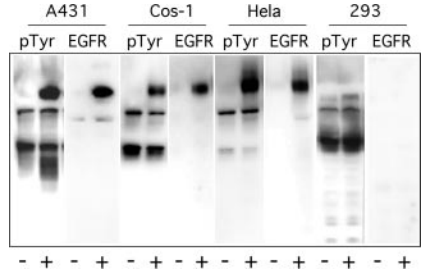

FIG. 6. **Immunoblots of cell lysates used in Figs. 4 and 5.** EGF-untreated (−) and -treated (+) cell lysates were separated by 4–20% gradient SDS-PAGE and transferred to nitrocellulose membranes. Phosphotyrosine (*pTyr*) proteins and activated EGFR were detected with anti-Tyr(P) and anti-active EGFR antibodies used in Figs. 4 and 5, respectively.

As shown in the Fig. 4, these cells responded differently to the EGF in terms of SH2 domain binding. EGF-induced SH2 domain binding was observed to be strong in A431 and HeLa

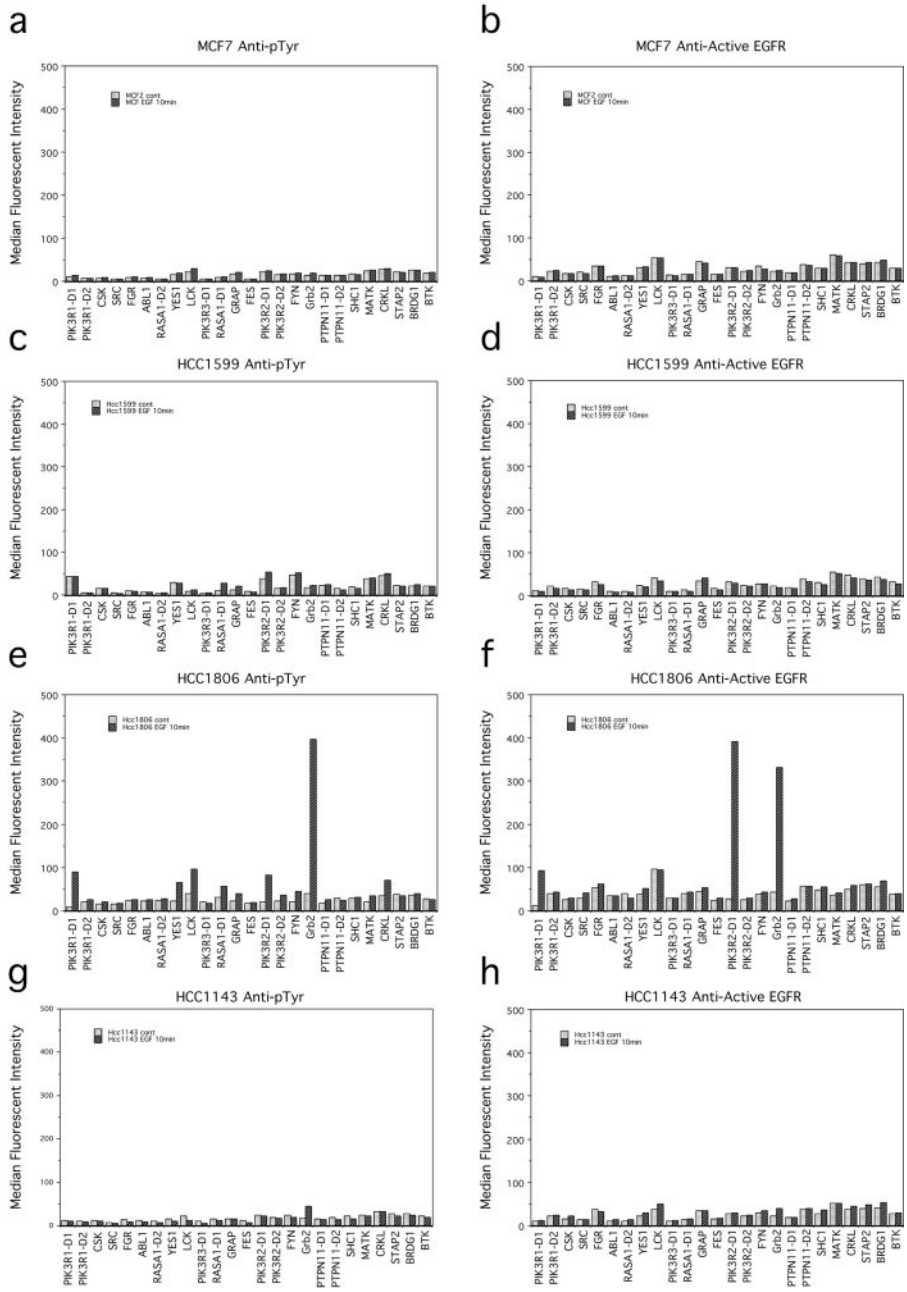

FIG. 7. **Profiling of EGF response of four breast cancer cell lines.** The breast cancer cell lines indicated were treated with EGF for 10 min prior to cell lysis, and then the cell lysates were assayed with 25-plex SH2 domain microsphere sets detected by anti-Tyr(P) (*pTyr*) antibody or anti-active EGFR antibody. Background signals for each region obtained from the assay with no cell lysates were less than 50 MFI. *a* and *b*, MCF7 detected with anti-Tyr(P) and anti-EGFR antibodies, respectively. *c* and *d*, Hcc1599 detected with anti-Tyr(P) and anti-EGFR antibodies, respectively. *e* and *f*, Hcc1806 detected with anti-Tyr(P) and anti-EGFR antibodies, respectively. *g* and *h*, Hcc1143 detected with anti-Tyr(P) and anti-EGFR antibodies, respectively.

and moderate in COS-1 cells, respectively, whereas very weak binding was observed in 293 cells. In A431 cells, the bound phosphoproteins were detected on PIK3R1 (also known as p85α) SH2 domain 1; PIK3R2 (also known as p85β) SH2 domain 1; RASA1 (also known as RASGAP) SH2 domain 1; and YES1, LCK, CSK, FYN, Grb2, MATK, and CRKL SH2 domains. Similar patterns of SH2 domain binding were observed in HeLa cells but not in COS-1 cells. The similar patterns between A431 and HeLa cells were observed at 5 min of EGF treatment (Fig. 4*b*). Interestingly at 30 min the binding was drastically diminished in HeLa cells (Fig. 4, *b* and *d*).

We also examined the binding of active EGFR to SH2 domains with antibodies against active EGFR using the same cell lysates as described above (Fig. 5). Grb2, PIK3R1 SH2 domain 1, and PIK3R2 SH2 domain 1 all strongly bound to EGFR only after EGF treatment in HeLa and COS-1 cells (Fig. 5, *c* and *d*). The binding observed in HeLa cells increased after 5 min of EGF treatment and then decreased by 30 min, whereas in COS-1 cells EGFR binding gradually increased, reaching the same level in 30 min that was observed after 5 min of treatment of HeLa cells. In A431 cells treated with EGF, the EGFR showed 5–10 times stronger binding to the three SH2 domains when compared with that in HeLa or COS-1 cells

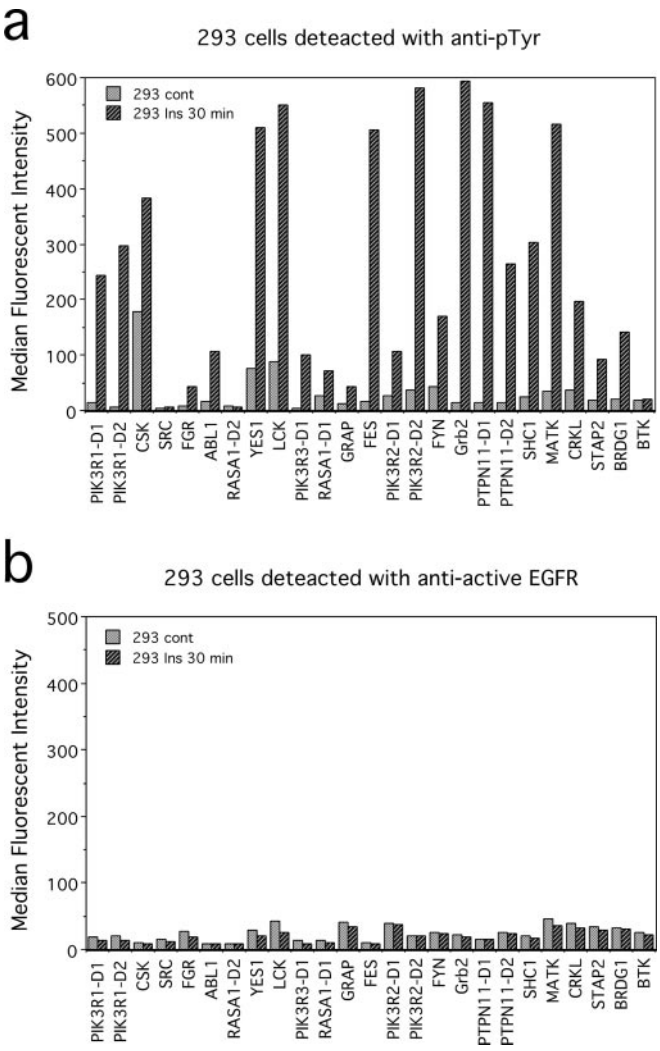

FIG. 8. **Profiling for insulin response of 293 cell line.** Cells treated with insulin (*Ins*) for 30 min were assayed with anti-Tyr(P) or anti-active EGFR using the 25-plex SH2 domain microsphere system. Background signals for each region obtained from the assay with no cell lysates were less than 50 MFI. *a* and *b*, detected with anti-Tyr(P) and anti-EGFR antibodies, respectively.

(Fig. 5*b*). EGFR binding in A431 cells reached a high level after 5 min of treatment and remained at that level after 30 min of EGF treatment. These cell lysates were also confirmed by immunoblotting with anti-active EGFR, and the results were consistent with those from the microsphere-based assay (Fig. 6).

Altered EGFRs are often indicated in breast cancers (13). We profiled the response of the breast cancer cell lines Hcc1806, MCF7, Hcc1599, and Hcc1143 to EGF treatment using the 25-plex microsphere assay to measure EGFR and phosphotyrosine protein binding to SH2 domains (Fig. 7). Only the Hcc1806 cells showed a significant EGF response, and the SH2 binding pattern was similar to that of similarly treated COS-1 cells (Fig. 4*c*). The cell line-dependent EGFR activation was also confirmed by immunoblotting with anti-active EGFR (data not shown).

Assay for Profiling Insulin-induced Activation of Phosphoproteins—To determine whether the 25-plex SH2-GST microsphere assay system is widely applicable for characterizing phosphotyrosine proteins regulated by other RTKs, we profiled phosphotyrosine protein activation in cell lysates following treatment with another cell stimulus, insulin, using the microsphere assay. Cell lysates from the commonly used 293, A431, COS-1, and HeLa cell lines treated with 100 nM insulin for 30 min were profiled by the 25-plex SH2-GST microsphere assay system. Only 293 cells showed a strong response to insulin as measured by the SH2 domain binding to cellular phosphoproteins, and the pattern of interactions was completely different from that seen for EGF-mediated activation (Fig. 8). The insulin-induced activation of 293 cells was confirmed by immunoblotting with anti-phosphotyrosine antibodies (data not shown).

DISCUSSION

As shown under "Results," both synthetic phosphotyrosine peptides and cellular phosphotyrosine proteins could be profiled using the highly sensitive and specific microsphere assay methods presented here. The GST-SH2 fusion proteins conjugated to the microspheres retained their functional binding activity to target phosphotyrosine peptide and did not cross-react with target peptides belonging to other domain families (Fig. 2). For the screening of the phosphotyrosine peptides (Fig. 3), we observed highly sequence-specific binding patterns at concentrations as low as 5 nM with this assay. Because this methodology utilizes a multiplexed high throughput format, it would be suitable for the large scale screening of small molecules, such as phosphopeptides, that specifically bind to the target SH2 domain(s) and inhibit SH2 domain-mediated protein-protein interactions. This would be of great utility for primary screening of new drug candidates.

In the assays of EGF-treated cell lysates, significant activation of EGFR in A431 cells was observed, supporting the findings of a previous report that A431 epidermoid carcinoma cells constitutively express EGFR at unusually high levels (22). HeLa cells responded rapidly to EGF, and the EGF-induced activation of SH2 interactions was drastically decreased after 30 min of treatment in agreement with the previous reports (23). The pattern of SH2 domain interactions, which indicates specific tyrosine residue phosphorylation of target proteins associated with each SH2 domain, differed slightly between various cell lines including the breast cancer cells. However, the overall patterns of SH2 domain interactions in EGF-responsive cells were similar to those observed previously in the EGF-induced activation of proteins downstream of the RTK (16). Insulin was used to test whether the assay system can be universally applied to profiling other RTK-activated signaling pathways, and we observed binding patterns that were significantly different from those of the EGF-treated cells. From these results, we concluded that the methodology presented

here is both sensitive and specific enough to distinguish between cell lines following treatment with different stimuli to activate specific RTKs.

The advantage of conjugating SH2 domains rather than phosphoprotein-specific antibodies to the microspheres is that one can detect novel binding partners for each SH2 domain under various conditions in the cell. In addition, if an antibody for specific protein of interest is available, the binding partner for the protein of interest can be simply profiled by the methods presented here. Also purified novel proteins can be tested to elucidate any possible tyrosine phosphorylations that are recognized by specific SH2 domain(s). On the other hand, because this assay relies on the binding of a second reagent (*e.g.* anti-Tyr(P) or antibody to a specific phosphoprotein) for detection, the use of this assay to specifically identify activated phosphorylated proteins may be problematic in some instances. Singly phosphorylated proteins will be undetectable when using anti-Tyr(P) for detection, whereas binding of large multiprotein complexes may yield misleading results with specific detection antibodies. In these instances, after profiling the SH2 binding patterns with the microsphere assay, one could identify each binding partner using a pull-down assay followed by two-dimensional gel electrophoresis and/or MS analysis.

We previously developed a membrane-based array system in which multiple SH3 domains are spotted to profile target ligands (24). While we prepared the manuscript, we noticed that a glass slide-based protein microarray, which carries over 100 SH2 domains, was reported in which synthetic peptides were used for profiling the binding of SH2 domains (25). Although the array systems can process only one sample per one membrane, the systems have an advantage over the microsphere-based assay because, in theory, large numbers of the various domains can be analyzed on a single array. Although Luminex microspheres containing 100 different regions are now available, we found the 25-plex format to be a realistic application of this technology because of potential cross-reactions, effects on specificity and sensitivity, and the costs of optimization and fluorescent dye labeling with more complex formats. Nevertheless the main advantage of the fluorescent microsphere-based technology is its multiplexed high throughput format with its ability to analyze ~96 samples in 3.5 h. To target all the SH2 domains found in the human genome, we plan to construct five sets of microspheres in the 25-plex format. With these assays, phosphotyrosine proteins that bind to genome-wide SH2 domains can be profiled; this will greatly facilitate the elucidation of underlying cellular signaling pathways with particular significance for use with drug screening.

Acknowledgments—We thank S. Han, J. Chen, R. Lam, and L. Roth for technical assistance and A. Han for editing the manuscript. We also thank C. Lai for helpful discussion in cell biology.

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S The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.

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