Profilers the Global Tyrosine Phosphorylation State*

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Protein tyrosine kinases and protein tyrosine phosphatases play a key role in cell signaling, and the recent success of specific tyrosine kinase inhibitors in cancer treatment strongly validates the clinical relevance of basic research on tyrosine phosphorylation. Functional profiling of the tyrosine phosphoproteome is likely to lead to the identification of novel targets for drug discovery and provide a basis for novel molecular diagnostic approaches. The ultimate aim of current mass spectrometry-based phosphoproteomic approaches is the comprehensive characterization of the phosphoproteome. However, current methods are not yet sensitive enough for routine detection of a large percentage of tyrosine-phosphorylated proteins, which are generally of low abundance. In this article, we discuss alternative methods that exploit Src homology 2 (SH2) domains for profiling the tyrosine phosphoproteome. SH2 domains are small protein modules that bind specifically to tyrosine-phosphorylated peptides; there are more than 100 SH2 domains in the human genome, and different SH2 domains bind to different classes of tyrosine-phosphorylated ligands. These domains play a critical role in the propagation of signals in the cell, mediating the relocation and complex formation of proteins in response to changes in tyrosine phosphorylation. We have developed an SH2 profiling method based on far-Western blotting, in which a battery of SH2 domains is used to probe the global state of tyrosine phosphorylation. Application to the classification of human malignancies suggests that this approach has potential as a molecular diagnostic tool. We also describe ongoing efforts to modify and improve SH2 profiling, including the development of a multiplexed assay system that will allow high-throughput functional profiling of the tyrosine phosphoproteome. Molecular & Cellular Proteomics 2:215–233, 2003.

Over the past two decades, it has become clear that tyrosine phosphorylation plays a pivotal role in a variety of important signaling pathways in multicellular organisms. In the typical vertebrate cell, phosphotyrosine (pTyr) represents only a tiny fraction of total protein phosphorylation (~0.05%). The presence of a constitutively activated protein tyrosine kinase (PTK) oncogene product might increase this percentage 10-fold (to ~0.5% of the total), and this relatively minor change is sufficient to induce malignant transformation (1). Because unregulated PTK signaling causes a breakdown in the normal regulation of processes such as cell proliferation and motility, leading directly to human diseases including cancer (2), tyrosine kinase signaling pathways are now a major focus of biomedical research.

Given the importance of tyrosine phosphorylation, a major challenge is to develop the means to rationally control and manipulate the cellular tyrosine phosphorylation state. The potential benefits are clearly illustrated by the remarkable success of the small molecule drug Imatinib (Gleevec, STI571; Novartis, Basel, Switzerland) in treating chronic myelogenous leukemia and other malignancies (3). Chronic myelogenous leukemia is caused by a chromosome rearrangement leading to the expression of a constitutively active PTK, the BCR-Abl fusion protein, which is strongly inhibited by Imatinib. A number of other agents that target PTKs are also in various stages of development; for example Trastuzumab (Herceptin; Genentech, South San Francisco, CA), an inhibitor of HER2/Neu/Erb2 receptor-type tyrosine kinase, has shown some success in combination with other anticancer agents in treating advanced HER2-overexpressing breast cancers (4). These success stories have clearly validated the usefulness of specific PTK inhibitors for treating human disease and also provide a persuasive justification for the identification of downstream effectors of PTK signaling, which may be expected to include novel therapeutic targets.

1 The abbreviations used are: pTyr, phosphotyrosine; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; MS, mass spectrometry; SH2, Src homology 2; pSer, phosphoserine; pThr, phosphothreonine; 2DE, two-dimensional gel electrophoresis; 1D, one dimensional; IP, immunoprecipitation; GST, glutathione S-transferase; IMAC, immobilized metal affinity chromatography; ICAT, isotop-coded affinity tags; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PhIAF, phosphoprotein isotope-coded affinity tags; phospho-Ab, phosphorylation site-specific antibody; PTB domain, phosphotyrosine binding domain; GSH-HRP, glutathione-horseradish peroxidase conjugate; PDGF, platelet-derived growth factor; PI3-kinase, phosphatidylinositol 3-kinase; PLCγ, phospholipase Cγ; GAP, GTPase-activating protein; RA, retinoic acid; MM, multiple myeloma; GIST, gastrointestinal stromal tumor; PBMC, peripheral blood mononucleated cells; AML, acute myeloid leukemia; SELDI-TOF, surface enhanced laser desorption/ionization time-of-flight; PCR, polymerase chain reaction.

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How should we best explore the downstream target molecules in PTK or protein tyrosine phosphatase (PTP) signaling pathways? Recent technical advances, including the availability of the complete human genome sequence, have set the stage for comprehensive or global analyses of PTK/PTP signaling. There are two broad approaches to this goal. One is the comprehensive identification of all PTKs/PTPs existing in the human genome, followed by elucidation of their function and regulation in the cell. Manning et al. have termed the full complement of protein kinases the “kinome,” and they currently estimate there are 90 individual PTKs in the human genome (5). Also taking into consideration the number of PTPs in the genome, the spatiotemporal regulation PTK/PTP activity is sure to be extremely complex. Therefore, the comprehensive profiling of these enzymatic activities in vivo would be an enormous challenge at the moment, although activity-based probe technology has begun to emerge as a promising tool for such studies (6, 7).

A second direction is the comprehensive identification of all tyrosine-phosphorylated proteins in the cell, the tyrosine phosphoproteome. This may be a more realistic goal, in part due to the availability of tools such as pTyr-specific antibodies (anti-pTyr) that can be used to detect or enrich for tyrosine-phosphorylated proteins. Historically, major tyrosine-phosphorylated proteins such as FAK, paxillin, p130Cas, etc. have been identified as prominent substrates in cells transformed by PTK oncogenes (8–10), implying biological relevance to transformation parameters such as cell growth, morphological alteration, or adhesion/motility, and indeed their biological importance has been validated in many cases (11–13). In contrast, the comprehensive detection of phosphoproteins must be unbiased. Furthermore, low-abundance proteins (or those phosphorylated at relatively low stoichiometry) are likely to play critical roles in vivo. Therefore, in order to explore new molecular targets in PTK/PTP signaling, reliable technologies that are both highly sensitive and selective are clearly needed.

To address this challenging problem, we have taken advantage of Src homology 2 (SH2) domains to develop a strategy for profiling the global tyrosine phosphorylation state (14). The SH2 domain is a small modular protein domain that binds specifically to tyrosine-phosphorylated peptide ligands; it is the most prevalent type of tyrosine phosphorylation binding motif in the cell, found in a large number of different proteins in metazoan organisms (15, 16). Because these domains play a critical role in normal signaling by mediating the formation of protein-protein complexes in response to changes in tyrosine phosphorylation, the SH2 binding pattern is likely to reflect functionally relevant aspects of the PTK signaling state. In the first part of this article, advantages and disadvantages of current mass spectrometry (MS)-based approaches for analysis of the phosphoproteome are discussed. In the next section, the SH2 domain assay (SH2 profiling) will be described, and finally prospects for the development of high-throughput SH2 profiling formats will be discussed.

**PHOSPHOPROTEOMIC APPROACHES**

The detection, identification, and quantitation of phosphoproteins, and mapping of their phosphorylated sites, are the fundamental aims of phosphoproteomics. Practically, phosphoproteomic approaches can be evaluated by how many phosphoproteins, especially low-abundance proteins, are identified from complex samples such as whole-cell protein extracts. Current MS-based phosphoproteomic approaches are outlined in Table I. While the downstream MS analysis obviously plays a key role in the output of each phosphoproteomic approach, sample preparation/purification approaches will be considered first, followed by a brief overview of MS technologies. Extensive reviews and protocols for current MS-based phosphoproteomic methods are available and should be consulted for further details (17–20).

**Two-dimensional Gel Electrophoresis**—Two-dimensional gel electrophoresis (2DE) is a standard tool for proteomics. However, given the low abundance of phosphoproteins, particularly those containing phosphotyrosine, this is not an efficient approach unless phosphopeptides can be enriched or specifically labeled. In a few cases, however, 2DE was successfully used in combination with functional information for analysis of phosphoproteins (21, 22). For example, Lewis et al. identified novel proteins involved in mitogen-activated protein kinase pathways by 2DE combined with MS based on the kinetics of change in abundance of spots in response to specific kinase activators (21).

**32P Labeling**—32P labeling is frequently used for phosphoproteomics as a highly selective and sensitive means of detecting phosphopeptides (23–25). Direct visualization and quantitation of phosphoprotein spots on 2D gels are possible with 32P labeling. Using high-resolution narrow-range 2DE, it is feasible to detect and quantitate differentially phosphorylated forms of a protein, which exhibit similar molecular mass but different isoelectric points (23). Moreover, 32P labeling coupled to immunoprecipitation (IP) allows phosphoproteomic analysis of particular protein complexes or organelles such as the 26S proteasome (26). However, the advantages of 32P are diminishing with the emergence of MS technologies that enable the direct detection of phosphoproteins as well as identification of the phosphorylation site. In addition, convenience and safety issues regarding the handling of radioactive materials are an unavoidable drawback of 32P labeling and preclude its use for human tissue samples.

**Immunoaffinity-based Methods**—IP with anti-pTyr is a powerful tool for enriching for low-abundance tyrosine-phosphorylated proteins, thereby improving sensitivity of detection in subsequent MS analysis. Anti-pTyr IP coupled to either onedimensional (1D) gel electrophoresis (20, 27–30) or 2DE (31–33) are now the most prevalent formats for tyrosine phosphoproteomic analysis, allowing unambiguous identification of tyrosine-phosphorylated proteins. Gel staining can be a crit-
**TABLE I**

MS-based phosphoproteomic approaches

Sample preparation/purification methods outlined here are all combined with MS-based peptide identification. For gel-based methods, stained gel bands or spots are excised, protease-digested, then analyzed by MS. In the case of Western blot-based techniques (nos. 8–11), the spot is excised from a reference gel corresponding to a spot of interest on the immunoblotted membrane. In chromatographic approaches (nos. 12, 14, 15–19), peptides are analyzed by MS without gel separation. In the table, “X” represents relative merit among the approaches with respect to following criteria; it should be noted that this information is derived entirely from published reports and future improvements are likely. Enrichment, enrichment of phosphorylated proteins/peptides, which may improve detection sensitivity; quantitation, accurate quantitation of relative phosphorylation level between different samples; site ID, unambiguous identification of phosphorylated residue(s); sensitivity, likelihood of detection of low-abundance proteins from a protein/peptide mixture; selectivity, likelihood of functional selection of specific proteins; throughput, the ability to handle large numbers of samples rapidly; pTyr, successful detection of tyrosine-phosphorylated proteins/peptides in the studies referenced here. 1, 2D gel, protein lysate separated by 2DE without any enrichment for phosphoproteins; 2, 32P-labeled gel, phosphopeptides are labeled with 32P, in vivo and separated by 2DE; 3, 32P-IP-2D gel, phosphopeptides are labeled with 32P, in vivo and lysates are immunoprecipitated with antibody to protein or larger complex (e.g. 26S proteasome (26)), then subjected to 2DE; 4 and 5, pTyr-IP-1D/2D gel, cell lysate is immunoprecipitated with anti-pTyr prior to 1DE or 2DE; 6, pSer/Thr-IP-1D gel, IP is done with anti-pSer/Thr antibody prior to 1DE; 7, IP/pulldown-1D gel, phosphoprotein is purified by binding with phosphopeptide recognition module (e.g. myc-tagged 14-3-3 protein (35) or GST-SH2 (34, 36)), then separated by 1DE; 8–10, 2D Western, 2D gel is transferred to membrane and immunoblotted with anti-pTyr (no. 8), anti-pSer (no. 9), or kinase substrate-specific antibody (no. 10); 11, 2D far-Western, far-Western analysis is performed using phosphoprotein binding probe (e.g. GST-fused substrate trapping mutant probe derived from catalytic domain of PTP1B tyrosine phosphatase (67)); 12, IMAC, phosphopeptides are enriched using IMAC (42–46); 13, 1D/2D gel-IMAC, isolated band/spot from 1DE/2DE gel is protease-digested and applied to nano-scale IMAC to facilitate identification of phosphorylation site; 14, pTyr-IP-IMAC, enrichment of tyrosine-phosphorylated proteins by anti-pTyr IP prior to IMAC; 15, 3H-IMAC, experimental sample and control are labeled in vivo with different isotopes prior to IMAC to quantitate phosphorylation changes; 16, biotinylation, Ser/Thr-phosphorylated proteins are β-eliminated, biotinylated, and purified by avidin affinity chromatography, and biotin tags enable unambiguous identification of phosphorylation sites by MS/MS (49); 17, sulfhydryl trap, trypsinized Ser/Thr phosphopeptides are modified with free sulfhydryls, then purified with immobilized iodoacetic acid (50); 18, PhIAT, PhIAT employs differential isotopic labeling and biotinylation of pSer/pThr residues via multifunctional tag; 19, d5-EtSH labeling, Ser/Thr-phosphorylated proteins are tagged with ethanethiol (EtSH) or ethane-d5-thiol (d5-EtSH) by β-elimination in vitro and combined to quantitate the relative phosphorylation state by LC/MS analysis; 20, in vivo label-1D gel, cells are metabolically labeled with stable isotopes and then combined, and proteins are extracted, separated by 1DE/2DE, and analyzed by MS, then relative levels of phosphorylation are determined by the ratio of unphosphorylated and phosphorylated peptide in the two samples (34, 54); 21, NIT-1D gel, N-terminal isotope-encoded tagging (NIT) is performed by N-terminal modification with d5- or d5-propionyl tags, combined with alkaline phosphatase treatment, and MS analysis of a separated 1D gel band allows relative quantitation of phosphorylated peptides (55).

| Category             | No. | Feature                  | Advantages                                      | pTyr | Ref.       |
|----------------------|-----|--------------------------|-------------------------------------------------|------|------------|
| Conventional         | 1   | 2D gel                   | Enrichment                                     | X    | X          | 21, 22 |
|                      | 2   | 32P-2D gel               | Quantiication, Site ID                         | X    | X          | 23–25 |
|                      | 3   | 32P-IP-2D gel            | Sensitivity, Selectivity, Throughput           | X    |            | 26    |
| Immunofinity         | 4   | pTyr-IP-1D gel           |                                                 | X    | X          | 27–30 |
|                      | 5   | pTyr-IP-2D gel           |                                                 | X    | X          | 31–33 |
|                      | 6   | pSer/Thr-IP-1D gel       |                                                 | X    |            | 40    |
|                      | 7   | IP/pulldown-1D gel       |                                                 | X    | X          | 34–36 |
|                      | 8   | pTyr-2D Western          |                                                 | X    | X          | 37    |
|                      | 9   | pSer-2D Western          |                                                 | X    |            | 41    |
|                      | 10  | 2D Western               |                                                 | X    | X          | 67    |
|                      | 11  | 2D far-Western           |                                                 | X    |            |       |
| Metal affinity       | 12  | IMAC                     |                                                 | X    | X          | 42–46 |
|                      | 13  | 1D/2D gel-IMAC           |                                                 | X    | X          | 48    |
|                      | 14  | pTyr-IP-IMAC             |                                                 | X    | X          | 39, 60|
|                      | 15  | 3H labeling-IMAC         |                                                 | X    | X          | 39    |
| Chemical/isotopic    | 16  | Biotinylation            |                                                 | X    |            | 49    |
| modification         | 17  | Sulfhydryl trap          |                                                 | X    | X          | 50    |
|                      | 18  | PhIAT                    |                                                 | X    | X          | 51, 52|
|                      | 19  | d5-EtSH labeling         |                                                 | X    | X          | 53    |
|                      | 20  | In vitro label-1D gel    |                                                 | X    | X          | 34, 54|
|                      | 21  | NIT-1D gel               |                                                 | X    |            | 55    |

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because unmodified proteins can bind to and coprecipitate with phosphoproteins. Combining anti-pTyr immunoblotting with 2DE provides considerable detection sensitivity for tyrosine phosphoproteins. Phosphoproteins can be identified by MS analysis of gel spots excised from a reference gel, which correspond to spots detected by immunoblotting (22, 37–39). Although this approach can be sensitive for protein identification, in many cases the amount of protein in a spot is not sufficient for identification of the specific phosphorylation site.

Because antibodies for phosphoserine (pSer) and phosphothreonine (pThr) are generally not thought to have sufficient specificity or affinity for IP, enrichment of serine- or threonine-phosphorylated proteins by IP with those antibodies has not been widely used. Recently, however, Gronborg et al. demonstrated that an anti-pSer/pThr PKA substrate antibody was capable of enriching pSer/pThr-containing proteins, leading to the identification of a novel signaling molecule (40). In addition, two-dimensional Western blot analysis with an AKT kinase substrate antibody was also used for sensitive detection of phosphosubstrates (41). Therefore, improved pSer/pThr antibodies have the potential to play a larger role in the future.

Immobilized Metal Affinity Chromatography—Gel-based “off-line” approaches have inherently low throughput, because electrophoresis, staining, and spot-picking are relatively slow compared with MS analysis. Immobilized metal affinity chromatography (IMAC) is a chromatographic technique for phosphopeptide enrichment based on the affinity of Fe(III) or Ga(III) for the negatively charged phosphate group and can be used in an on-line high-throughput format; accordingly, it has been widely used for phosphoproteomic studies (42–46). Although IMAC purification is not absolute due to the binding of acidic peptides (19, 47), Ficarro et al. have reduced this background binding by methylester modification of carboxyl groups prior to IMAC, thereby improving detectability of phosphopeptides in subsequent MS (45). They could detect more than a thousand phosphopeptides and identified 383 phosphorylation sites in 216 peptides starting with 500 μg of yeast protein. This is outstanding sensitivity and throughput compared with other published reports. Stensballe et al. reported that custom-made nanoscale Fe(III)-IMAC columns, in combination with 2DE, increased the likelihood of identification of phosphorylation sites (48). Of course this off-line format is incompatible with an automated high-throughput system.

Chemical Modification and Isotopic Labeling—Tagging phosphopeptides by specific chemical modification is attractive because it is amenable to large-scale analysis. Methods in which the phosphate moiety is chemically modified, e.g. by biotinylation, allow enrichment of phosphopeptides by affinity chromatography and the subsequent unambiguous identification of the phosphorylated site (Table I, nos. 16–18) (49–52). On the other hand, methods in which peptides are differentially labeled with stable isotopes such as 12C/13C or 14N/15N allow accurate determination of the abundance of specific phosphopeptides in one sample relative to another by measuring relative MS signal intensity (Table I, nos. 15, 18–21) (34, 39, 51, 53–55).

The goal of the isotope-coded affinity tag (ICAT) method is to quantitate relative protein amounts in two samples without separation by 2DE. All cysteine residues in one sample are modified with a biotinylated “heavy” isotope tag, and those of a second sample with a similar “light” isotope tag; the two samples are then combined and the relative intensity of corresponding heavy and light peptides is determined by MS (56). Phosphoprotein isotope-coded affinity tags (PhIATs) is conceptually similar to ICAT, but pSer and pThr residues are tagged instead of cysteine residues. This permits the simultaneous enrichment, quantitation, and identification of phosphopeptides via a biotinylated isotope tag (51). While chemical modification methods based on the β-elimination reaction, including PhIAT, cannot modify tyrosine residues (Table I, nos. 16, 18, and 19), a different approach based on a carbodiimide condensation reaction can be applied to pTyr (Table I, no. 17) (50).

It has been pointed out that current chemical modification approaches can only detect relatively abundant phosphoproteins (19). Methods aimed at enriching phosphorylated peptides prior to modification have been somewhat disappointing, with increased sensitivity compromised by losses due to multiple additional purification steps. However, a recent report demonstrated the identification of low-abundance phosphoproteins by the PhIAT system (52).

Mass Spectrometry

There is no doubt that current MS technologies have enormous advantages over traditional Edman sequencing, both in sensitivity and throughput. For instance, Yoshimura et al. reported the identification of a total of 28 32P-labeled spots (potential substrates of Ca2+/calmodulin-dependent protein kinase II) by nanoflow liquid chromatography-tandem mass spectrometry (MS/MS) analysis, of which 12 were previously not detectable by Edman sequencing (25). Several approaches are currently used to detect phosphopeptides by MS (20). The matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS “peptide fingerprint” method is a standard tool for peptide identification. In this type of analysis, phosphorylated peptides can be detected by the characteristic 80-Da mass reduction upon alkaline phosphatase treatment. Collecting MS spectra from samples treated with different proteases increases the likelihood of detecting phosphopeptides (24). The lower sensitivity of detection for phosphopeptides in positive ion mode can be overcome by direct scanning of fragment ion PO4− in negative ion mode of MS/MS analysis. An m/z value of −79 for its fragment ion is specific and sensitive for its detection; however, this ap-
approach cannot differentiate between pSer, pThr, and pTyr, and subsequent peptide sequence analysis is needed with different polarity. Another approach takes advantage of precursor scanning for the immonium ion of phosphotyrosine (C₉H₁₀NO₄P), with m/z of 216.043. Using this method for specific detection of phosphotyrosine, coupled to pTyr-IP-1D gel, it is possible to identify a large number of known/un- known signal transduction molecules (27–29, 57). Undoubtedly, this MS approach will become a standard tool for tyro- sine phosphoproteomics. However, because the pTyr immonium ion (m/z 216.043) must be differentiated from other ions with similar nominal mass (e.g. C₉H₈NO₃S, m/z 216.069), an MS system with extremely high accuracy is required, such as a quadrupole time-of-flight hybrid tandem mass spectrometer (58, 59).

Comparison of Different Approaches

While a variety of different approaches for phosphoproteomics are currently available, it is difficult to assess their relative merits because of differences in the cell systems analyzed or downstream MS analysis in different studies, which may greatly affect sensitivity and data quality. In this regard, direct comparison of different approaches in the same system is invaluable, especially for the analysis of low-abun- dace tyrosine-phosphorylated proteins. Ficarro et al. compared multiple IMAC-based approaches using capacitated human sperm (39). A total of 200 distinct phosphopeptides were detected from the crude protein mixture, in which 60 phosphorylation sites were mapped. However, in this study only five pTyr sites were mapped, four of which were in peptides that also contained pSer, consistent with the notion that multiply phosphorylated peptides are preferably enriched by IMAC (19).

Immunoaffinity approaches employing pTyr-2D Western blot assays and pTyr-IP-IMAC were also compared in that study (39). Surprisingly, pTyr-2D immunoblotting was more sensitive than pTyr-IP-IMAC for detecting and identifying tyrosine phosphoproteins. This fact reminds us that the pTyr-2D Western blot assay is a powerful tool for tyrosine phosphoproteomics. Phosphoprotein identification by the combination of pTyr-2D Western blot assays and MALDI-TOF MS has become standard, but mapping the phosphorylation sites by this approach remains a challenge. In contrast, IMAC or chemical modification may increase the likelihood of unambiguos determination of the site, and these on-line approaches are well adapted for high-throughput large-scale analysis. Indeed, pTyr-IP-IMAC was recently used to map 64 known/unknown tyrosine-phosphorylated sites on 32 different proteins from human hematopoietic cells (60), and mapping of several hundred phosphorylation sites by IMAC has been reported (45).

In summary, it is obvious that specific approaches need to be chosen depending on the purpose. At the moment, the pTyr-2D Western blot assay is highly sensitive for detection, and low-abundance phosphoproteins can be sequenced by enrichment with anti-pTyr IP. In contrast, IMAC or stable isotope labeling may have the advantage for detecting phos- phorylation sites or the relative quantitation of phosphopep- tides, respectively. Importantly, for all of these approaches appropriate sample preparation prior to MS analysis is crucial for obtaining high-quality data (61).

Profiling Tyrosine Phosphoproteins

Like genomics, phosphoproteomics is an identification-based strategy, where the primary end point is the compre- hensive identification and quantitation of phosphoproteins in a specific cellular context. However, to facilitate the develop- ment of new diagnostic and therapeutic targets, the functional profiling of the phosphoproteome is essential (62, 63). Such functional data would include the identity of the phosphoryl- ated protein, the sites phosphorylated, and the kinetics of phosphorylation/dephosphorylation under various physiological conditions. As important, but undoubtedly more challeng- ing, would be characterization of the upstream enzymes res- ponsible for changes in phosphorylation, as well as the downstream consequences of changes in phosphorylation. At the moment, global phosphoproteomic profiling is technically difficult because the sensitivity of available methods is insuf- ficient to identify all PTK substrates in a given sample.

Even without the comprehensive identification of all phos- phorylated proteins in a sample, however, a great deal of useful information can be gleaned by profiling the phospho- rylation of specific proteins or specific classes of phosphoryl- ation sites. Phosphorylation site-specific antibodies (phos- pho-Abs), which can detect specific phosphorylated proteins by conventional Western blot assay, have become important tools for analyzing signaling pathways. The coupling of phos- pho-Abs to protein chip technologies based on high-through- put MS systems such as surface-enhanced laser desorption/ ionization time-of-flight (SELDI-TOF) MS may emerge as a valuable approach (64–66). Kinase-substrate antibodies that specifically detect individual kinase substrates are also a promising tool for phosphorylation profiling (41). However, generating phosho-Abs involves significant labor and cost, and those currently available do not recognize all known sites (and the number of unknown sites is presumably enormous), so it is not feasible to profile all the tyrosine-phosphorylated proteins in the cell by this approach.

SH2 PROFILING

Given this background, there is clearly an urgent need for a rapid method that can functionally profile the tyrosine phos- phorylation state of the cell. One promising approach takes advantage of the fact that most tyrosine-phosphorylated pro- teins serve as high-affinity binding sites for small protein interaction modules, namely SH2 domains and phospho-
tyrosine binding (PTB) domains (15, 16, 68, 69). Thus, PTK substrates may in principle be classified on the basis of the presence or absence of binding sites for specific SH2/PTB domains. Because the total number of SH2 and PTB domains is relatively modest, and different domains bind to different classes of tyrosine-phosphorylated sites, characterizing the binding sites for these domains can serve to efficiently profile the global state of tyrosine phosphorylation. This approach is obviously functionally relevant, in the sense that it relates directly to the functional properties of the phosphorylated sites detected, because the binding of SH2 and PTB domains actually plays a vital role in the course of normal signaling in the cell (16).

**Methodology**

SH2 domains are modular, and the recombinant domains can easily be produced in bacteria and bind well to their tyrosine-phosphorylated ligands in vitro; these properties suggested that SH2 domains could be used to probe the tyrosine phosphorylation state of a protein sample. We use the term “SH2 profiling” to describe methods in which a battery of SH2 domain probes are used to provide a qualitative and quantitative fingerprint, or profile, of the overall state of tyrosine phosphorylation (14). In the current method based on the far-Western filter binding assay, a protein extract is separated by conventional 1D gel, transferred to a nitrocellulose or polyvinylidene difluoride membrane, and subsequently probed with labeled GST-SH2 fusion proteins. Replicate filters are probed with different SH2 domains having different binding specificities, and the pattern of binding (intensity and apparent molecular mass of specific bands) for each probe can be compared among different samples.

One general concern with far-Western blotting is the ability to detect specific signals above the background of nonspecific binding. This is a particularly important issue for detection of low-abundance binding partners such as tyrosine-phosphorylated proteins in normal cells. We found that specific signal was enhanced and background reduced when glutathione-horseradish peroxidase conjugate (GSH-HRP) was used to label the GST-SH2 fusion protein probe, as compared with standard detection methods including anti-GST antibody or direct biotinylation of the GST fusion protein (14). While we have not exhaustively examined the reasons for this, one possible explanation is the fact that GSH-HRP can bind only to native, functional GST; therefore, any denatured GST fusion protein, which might bind nonspecifically to proteins on the filter, is not labeled. Another advantage is rapidity; because there is no need to incubate with secondary detection agents (2° antibody, streptavidin conjugate, etc.), washing and incubation times are minimized. Indeed, a 10-min binding reaction followed by brief washing is often sufficient. Finally, the GSH-HRP conjugate is highly oligomerized, containing large numbers of GSH and HRP moieties per complex; thus, incubation of GSH-HRP with GST-SH2 fusions generates a probe with very high avidity of binding (because it has many copies of the SH2 domain) and high specific activity (because it has many HRP molecules), thereby increasing the specific signal.

A central premise of this approach is that different SH2 domains maintain their phosphorylation-dependent and sequence-dependent binding specificities in the far-Western filter binding assay. The phosphorylation dependence of binding has been confirmed by tyrosine phosphatase treatment of the membrane, which led to complete loss of SH2 domain binding (14). Peptide sequence specificity was also addressed. When binding was performed with a single SH2 domain probe at moderate concentrations (e.g. 1 µg/ml), specificity was in some cases compromised; this is most likely because the probe was present in great excess relative to the phosphorylated targets on the membrane, leading to some nonspecific binding to suboptimal sites. We found that specificity could be dramatically improved by performing binding under competitive conditions, where a single labeled SH2 domain competes in the binding reaction with multiple unlabeled heterologous SH2 domains (14). When GSH-HRP is used for detection, however, competitors must be chemically modified to ensure that they cannot bind GSH (otherwise the label would equilibrate between the probe and “competitors”). This can be easily accomplished by treatment of competitors with ethacrynic acid, a GST inhibitor that stably binds to the GSH binding site of GST (14).

In experiments using GSH-HRP-labeled SH2 domain probes and ethacrynic acid-blocked competitors, we showed that SH2 domains could detect specific tyrosine-phosphorylated sites in a protein expressed at endogenous levels in a whole-cell protein lysate. In cells expressing either wild-type platelet-derived growth factor (PDGF) β receptors or mutants in which specific SH2 binding sites were eliminated, the specificities of the phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase (PI3-kinase), and p120 Ras-GTPase-activating protein (GAP) SH2 domain probes were tested. As expected, each SH2 domain detected the wild-type PDGF receptor when autophosphorylated in response to PDGF treatment. Each SH2 domain probe no longer bound, however, when the tyrosine residue corresponding to its known binding site on the receptor was mutated (14). This clearly demonstrates the feasibility of using SH2 profiling to characterize PTK signaling: the assay has both the specificity to discriminate among different tyrosine-phosphorylated sites and the sensitivity to detect phosphorylation of a single site on a signaling protein expressed at relatively low levels.

It is important to consider several practical issues regarding the sensitivity and specificity of the SH2 profiling approach. We have shown that in many cases binding of SH2 domains can visualize bands that are not apparent by standard anti-pTyr immunoblotting. Anti-pTyr is accepted to be a very sensitive technique for detecting tyrosine-phosphorylated pro-
teins; thus, we can conclude that the sensitivity of SH2 profiling is as good if not better. In competitive binding experiments, however, the presence of unlabeled competitor SH2 domains affects both sensitivity and specificity. Increased binding specificity under competitive conditions is accompanied by a significant decrease in overall signal intensity compared with the noncompetitive situation. Therefore, for some SH2 domains, especially those with relatively low affinity and/or high intrinsic specificity, noncompetitive binding conditions can give superior results. The mix of competitor SH2 domains must also be carefully considered. Ideally, the competitors will include many SH2 domains with different binding specificities, but not domains closely related to the labeled probe, which would be expected to have similar binding specificity and thus diminish the specific signal through direct competition. The mixture of competitors that maximizes specificity and signal strength may vary for each probe and must be determined empirically. However, we have found that a “standard” competitor mixture of 5–10 SH2 domains can be used successfully for many probes.

Applications

We have used the SH2 profiling approach to study a variety of biological systems. In one example, we have profiled changes in tyrosine phosphorylation patterns in the course of neuronal differentiation in a cell culture system where PTK signaling has been suggested to play an important role (70, 71). Fig. 1A shows the morphological changes in p19 embryonal carcinoma cells that have been induced to differentiate in response to retinoic acid (RA). RA-treated cells initially withdraw from the cell cycle and become more tightly adherent, then after 10–14 days they begin to display neuron-like morphology (72, 73). The tyrosine phosphorylation state was determined by anti-pTyr Western blot and by the far-Western SH2 profiling assay (Fig. 1B) in the course of RA-induced differentiation. The binding of three representative SH2 probes, whose binding patterns were clearly distinct, were superimposed and represented in different colors (right panel of Fig. 1B).

The binding patterns for the PI3-kinase, Crk, and GAP SH2 probes show distinct kinetic changes during the course of differentiation, demonstrating that different proteins with different SH2 binding properties become differentially phosphorylated as cells differentiate. It is particularly striking that nearly all of the bands detected in the anti-pTyr blot are represented in the superimposed SH2 profile, but the SH2 profile “de-convolutes” the pattern in terms of what class of tyrosine-phosphorylated sites are present in each band. Thus, SH2 profiling gives similar information in terms of the number of bands and their molecular mass, but provides much more information regarding the nature of phosphorylated sites than anti-pTyr blotting alone.

We further asked whether this approach could serve as a basis to classify human malignancies based on their tyrosine phosphorylation state. There is currently great interest in developing novel molecular diagnostic methods that can classify tumor cells in biologically and clinically relevant ways. In particular, classification methods that have predictive value in terms of the course of disease, response to therapy, and other clinical parameters will be of great value in choosing the best treatment options for each tumor. cDNA expression profiling has been proposed as a method for both class identification and class assignment and has shown some promise in pilot studies (74–76). Because tyrosine phosphorylation can play a critical role in tumorigenesis, as well as regulating factors important for progression including angiogenesis and motility,
we reasoned that a global fingerprint or profile of the tyrosine phosphorylation state of a tumor might serve as a functionally relevant means of classifying otherwise similar tumors. Consistent with this idea, SH2 profiling of various human cancer cell lines including prostate, breast, and colon cancer demonstrated that each cell line has a distinct SH2 binding profile (Fig. 2). Furthermore, some cell lines of similar origin (for example the CX-1 and DLD1 colon carcinoma cell lines) show similar patterns, while others are quite distinct.

Multiple myeloma (MM) is a B cell neoplasm characterized by the accumulation of clonal plasma cells in the bone marrow. The frequent occurrence of resistance to chemotherapeutic agents has been a major issue at the bedside, and it would be of great use to identify markers that correlate with drug resistance. We profiled a number of MM cell lines varying in their resistance to drugs used in conventional chemotherapy for MM; the availability of sensitive and resistant lines derived from the same tumor allows a controlled comparison of changes that may be associated with resistance to particular drugs. As shown in Fig. 3A, SH2 domains are capable of discriminating between highly related MM cell lines that differ in their resistance to anticancer agents. This finding implies that tyrosine-phosphorylated proteins may serve as markers of drug resistance and further raises the possibility that the actual mechanism of resistance may in some cases involve tyrosine phosphorylation. We are now further characterizing candidate phosphoproteins to test this possibility. Obviously further studies using large numbers of primary human patient samples would be required to establish any significant correlation between SH2 binding patterns and drug resistance, but these pilot results suggest at least that this approach can identify reproducible differences in very closely related but functionally distinct tumor cells.

We have also applied SH2 profiling to gastrointestinal stromal tumors (GISTs). GIST is the most prevalent type of human mesenchymal tumor of the gastrointestinal tract, more than 90% of which have activating mutations in the c-Kit receptor tyrosine kinase. Recently, the specific PTK inhibitor Imatinib (STI571) has been used to treat GIST patients, demonstrating dramatic clinical response (77, 78). We have profiled several GIST cell lines including primary tumors, and one of these was compared in the presence or absence of Imatinib. The overall SH2 profiles of these GIST cell lines were dramatically different, again illustrating the potential of this approach in class identification and class assignment (data not shown). Remarkably, among more than 10 different SH2 domain probes tested, only the binding of the PI3-kinase SH2 probe was dramatically decreased upon Imatinib treatment (Fig. 3B, middle panel, and data not shown). This result is consistent with the functional involvement of PI3-kinase signaling pathways in Imatinib-sensitive human malignancies (79, 80). Imatinib reduced the phosphorylation of multiple proteins in these cells; interestingly, superimposition of c-Kit Western and PI3-kinase SH2 far-Western data demonstrates that while the binding of the PI3-kinase SH2 to the autophosphorylated c-Kit receptor (the direct target of Imatinib) is decreased by the drug, the receptor is a relatively minor PI3-kinase SH2 binding protein. Identification of the major Imatinib-sensitive PI3-kinase SH2-binding proteins (between 100 and 120 kDa) may reveal important downstream mediators of c-Kit signaling in GIST.

We are also testing the feasibility of using SH2 profiling to classify clinical samples from patients with various cancers. We chose hematopoietic malignancies for initial studies because of the ease in obtaining tumor cells from blood and bone marrow; however, similar studies are now underway for solid tumors as well. While the number of samples analyzed to date is too small to indicate whether profiling results will have any prognostic value, these pilot studies have demonstrated that clear and reproducible profile data can be obtained from patient samples and that different tumors of the same diagnostic class can have grossly different profiles. We have noticed, however, that clinical specimens tend to have somewhat weaker signal levels relative to cultured cell lines, so care must be taken to optimize signal-to-noise ratios when analyzing such samples.

Fig. 4 shows representative profile data obtained from clinical samples, in this case from two patients with acute myeloid leukemia (AML; FAB classification M2). In both cases, bone marrow as well as peripheral blood mononucleated cells (PBMC) were analyzed for each patient. Several observations are relevant in the context of molecular diagnostics. First, the SH2 profiles of the PBMC samples from the two patients are clearly distinct, despite the similar pathological classification of the malignancies. Second, the profiles for the PBMC and bone marrow of patient 1 are clearly very similar, as expected if the same tumor cells are present in both blood and marrow. This (and other data not shown) demonstrates that SH2 profiles are reproducible from sample to sample, despite differences in preparation. In contrast, very little signal is seen in the bone marrow from patient 2, consistent with the clinical
diagnosis of bone marrow remission for this patient at the time the sample was taken. Taken together, these results suggest it is likely that SH2 profiling will provide novel information that can be used to classify tumors and may even serve to identify novel diagnostic markers or targets for therapy.

Practical Considerations and Other Formats

**Data Analysis**—Data from SH2 profiling experiments can be analyzed in several different ways. 1D gel band patterns or “fingerprints” for a number of SH2 domains can be digitally acquired for each sample and normalized (81). This permits the overall patterns for different samples to be compared and classified based on quantitative similarities in binding pattern, and statistical analyses can be applied to identify potentially significant correlations between binding pattern and biologically or clinically relevant parameters (response to therapy, survival, etc.). While technically feasible, we have not yet established optimal automated protocols for normalizing and comparing profile data because the number of samples to date has been relatively modest. Instead, we manually generate images in which the binding patterns for individual SH2 domains are rendered in false color and superimposed (Figs. 1–4). For this process, and indeed for any method of archiving and comparing SH2 profiling data, it is critical to use standardized and reproducible conditions for gel electrophoresis and membrane transfer. Commercially available precast gels and blotting methods that generate multiple membranes from a single gel will be useful in this regard. It should be noted that stripping and reprobing of membranes is possible, but often results in loss of signal intensity and increased background (data not shown). Manual image analysis is adequate to identify major differences and similarities between samples, but obviously automated computer-based methods for data analysis are an important priority for large-scale quantitative studies.

**Protein Identification**—While differences in the overall pattern of SH2 binding will be useful for class identification and class assignment, the unambiguous identification of the proteins corresponding to specific bands will be important for understanding the biological significance of profile data. An obvious approach for identification is to take advantage of SH2 domains as affinity reagents to purify binding proteins from a sample, then to identify the bound proteins (and specific phosphorylation sites) using standard MS-based methods. Given the low abundance of most tyrosine-phosphorylated proteins and the unfavorable solution binding kinetics that this implies, relatively large amounts of starting material...
(10^8–10^9 cells) are necessary. We are currently purifying SH2 binding proteins of interest by this approach, but have found that low-abundance phosphoproteins are sometimes undetectable. It is likely that the binding of phosphorylated peptides to endogenous SH2 domain-containing proteins present in the cell lysate and other steric factors contribute to making simple affinity purification with SH2 domains relatively inefficient. However, Blagoev et al. reported identifying 228 proteins from epidermal growth factor-stimulated cells by GST-Grb2 SH2 domain binding followed by MS analysis (34), so the combination of SH2 profiling and protein identification may evolve into a powerful tool for phosphoproteomics.

Relevance to Signaling Pathways—It is important to keep in mind that SH2 binding data can provide information about binding sites for endogenous SH2 domain-containing proteins present in the cell lysate and other steric factors contribute to making simple affinity purification with SH2 domains relatively inefficient. However, Blagoev et al. reported identifying 228 proteins from epidermal growth factor-stimulated cells by GST-Grb2 SH2 domain binding followed by MS analysis (34), so the combination of SH2 profiling and protein identification may evolve into a powerful tool for phosphoproteomics.

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It will also be very useful to begin identifying the components of SH2 domain-phosphoprotein complexes that are present in the cell, and to assemble databases of such complexes in various cells where SH2 profiling has identified potentially important differences in phosphorylation. For example, in the GIST example cited above, it would be of great interest to identify complexes containing phosphoproteins that bind the PI3-kinase SH2 domains. Such an approach has the potential to reveal upstream and downstream components in critical signaling pathways in the cell of interest. Several comprehensive studies have been published in which stable protein complexes have been identified by the combination of expression of a tagged protein of interest, followed by isolation of the tagged protein (along with binding partners) and subsequent MS analysis (82, 83). Similar approaches are feasible for identifying SH2 binding proteins in cell lines, and as discussed above direct affinity purification using SH2 domain probes may also be useful in this regard.

Binding Considerations—To maximize the information obtained from SH2 profiling experiments, ideally one would use a comprehensive panel of probes consisting of all SH2 domains with distinct binding specificities. We are currently using a total of 44 different SH2 probes for the SH2 profiling assay, derived from 24 different SH2 domain-containing proteins. This number is still far from the total number of SH2 domains existing in the human genome (at least 115). PTB domains also can bind to specific tyrosine-phosphorylated proteins (68, 69); the binding specificity and sensitivity of the only PTB domain we have tried to date, that of Shc, is sufficient for the far-Western assay (Fig. 3A). Thus, other phosphotyrosine-dependent PTB domains are good candidates for additional probes recognizing distinct classes of tyrosine-phosphorylated sites.

In the end, because of overlapping binding specificities we expect that the complete set of SH2/PTB domain probes will not be necessary to comprehensively sample all classes of tyrosine-phosphorylated binding sites. For example, the structure and binding specificity of the SH2 domains of the eight human Src family tyrosine kinases are very similar (84, 85), thus only one or two representatives from this family need be included in profiling studies. Similarly, many other SH2-containing proteins belong to closely related families with two or more members, only one of which need be included. SH2 domains may be further classified into subgroups according to sequence, three-dimensional structure, peptide screening

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FIG. 4. SH2 profiling of clinical samples from AML patients. White blood cells were purified from peripheral blood (lanes 1 and 3) or bone marrow (BM, lanes 2 and 4) by Percoll (Amersham Biosciences) according to manufacturer’s instructions. AML#1 and #2 were taken from two different patients, each clinically diagnosed as AML (FAB class M2). The two AMLs are clearly distinct in their overall SH2 profiles, whereas the blood and bone marrow samples for patient 1 are clearly similar. Patient 2 was in bone marrow remission at the time the sample was taken.
data, and far-Western binding data. Thus, in the future we anticipate a standard screening strategy using a representative panel of ~20 SH2 domains, chosen for distinct binding specificity and relatively strong signals and low background. Alternatively, the comprehensive set of all SH2 and PTB domains could be assayed using various high-throughput multiplexed assay formats outlined in the next section.

Although the competitive SH2 profiling assay allows highly specific detection of tyrosine-phosphorylated proteins, it is obviously not equivalent to protein-protein interaction in the cell, where SH2 domain-phosphopeptide interactions are tightly regulated by local concentration, other protein domains, and binding partners. Therefore, in vitro binding data must be interpreted thoughtfully. Each SH2 domain has a distinct spectrum of affinities for target phosphopeptides, with dissociation constants ($K_d$) for specific targets in the range of 100 nM to 1 μM (86). Thus, the binding of an SH2 domain probe that is highly specific but has relatively low affinity for its ligands might not be detected in vitro, where conditions have been optimized for high-affinity probes. We have therefore explored ways to improve the apparent affinity of some SH2 probes.

Multimerization of a protein binding module would be expected to increase the avidity of binding, and indeed “natural” tandem SH2 domains, such as those of SHP-2, Zap-70, and PLC\(\gamma\), have been shown to have greater avidity than single domains (87). Such avidity effects would be expected to be exaggerated in the solid-phase far-Western binding assay, where high local concentrations of a phosphorylated binding protein are present in a band on the membrane, allowing the multiple SH2 domains of a tandem probe to bind simultaneously. We have applied a recombinant tandem SH2 domain strategy to the c-Abl SH2, which is normally present in a single copy, and PLC\(\gamma\), which normally contains a tandem pair of SH2 domains. In each case, GST fusion proteins were constructed containing various numbers of tandemly repeated SH2 domains. In both cases the multimerized SH2 domain probes gave stronger binding signals without apparent loss of specificity. As shown in Fig. 5, significant improvement in signal intensity was observed for clinical samples, for which specific binding is often difficult to detect for some probes. It should be noted that all of our SH2 probes are fusions with GST, which exists as a dimer in solution, thus even single SH2 probes are in actuality homodimers. Consistent with the importance of multimerization, we have previously observed that SH2 domains cleaved from GST bind much more poorly than the corresponding GST fusions in far-Western assays (Ref. 14 and data not shown).

Other Formats—Given the major role played by 2DE-pTyr immunoblotting in phosphoproteomics, and reported successes with 2DE-far-Western proteomics (67), it will be of interest to apply SH2 profiling to a 2D gel format. Generating an SH2 domain-specific fingerprint by 2D far-Western blot assay would obviously provide more information than anti-pTyr 2D Western blotting, as SH2 binding provides additional information about the nature of specific phosphorylation sites. However, the well-known drawbacks of 2D gel analysis must be considered, including limitations on protein size, difficulties in resolving very hydrophobic, acidic, or basic proteins, and the relatively large amounts of protein required (150–300 μg per 2D gel versus 15–50 μg per lane of 1D gel). Moreover, because the 2D gel format has inherently low throughput (one gel per sample), it would be a challenge to routinely perform 2D SH2 profiling with multiple SH2 probes, as analysis of a single sample would require a large number of gels. Therefore, it is likely the 2D gel format will be useful in limited situations, for the detailed analysis of samples determined to be of particular interest by 1D profiling or other methods.

We are also exploring an alternative approach to SH2 profiling that can be applied to intact cells and tissues. As mentioned above, SH2 profiling can differentiate tumor cells based on their SH2 domain binding patterns; however, clinical specimens, especially solid tumors, generally contain a mixture normal and tumor cells that may themselves exhibit considerable heterogeneity. Furthermore, biopsy samples often contain only a small number of tumor cells, insufficient for analysis by the far-Western approach. Thus, a method allowing visualization of SH2 binding at the single-cell level would be quite useful, and we are therefore developing an immunohistochemistry-based method in which SH2 domain probes are used to stain the tumor cell directly.
Detection of phosphorylation in fixed, paraffin-embedded tumor sections is challenging, but successful staining by phosphospecific antibodies has been reported (88–91). We found that by modifying SH2 domain binding and staining conditions, we could also detect SH2 domain-specific signals from paraffin-embedded sections of breast carcinomas (Fig. 6). Of course unlike the far-Western binding assay, no information about the identity (molecular mass) or number of SH2-binding proteins is obtained by this approach. However, absolute quantitative binding information (strong binding, weak binding, no binding) for a large panel of SH2 domain probes is likely to provide sufficient information to classify tumor cells based on similarity in binding patterns. An obvious advantage of this approach is that archived samples linked to existing patient records can be analyzed, permitting large-scale retrospective studies to assess possible correlations between tyrosine phosphorylation patterns and clinical outcomes. Furthermore, quantitative data analysis is greatly simplified compared with the far-Western format. For clinical samples, SH2 profiling in both the 1D far-Western and tissue-staining formats may provide complementary data, representing protein-specific and cell-specific data, respectively.

**MULTIPLEXED ASSAY FORMATS FOR SH2 PROFILING**

While the far-Western-based SH2 profiling approach provides high sensitivity and specificity, it requires separate binding reactions for each SH2 domain probe. Given the number of SH2 and PTB domains likely to be needed to cover all binding specificities and thus obtain a comprehensive signaling profile, the far-Western blot approach is therefore inherently cumbersome and low throughput because many analyses must be performed in parallel. Moreover, the number of parallel analyses can be limited by the amount of sample protein available, especially in clinical situations such as the analysis of cancer cells from biopsies or microdissected tissue. To overcome the limitations of the far-Western blot approach, alternative high-throughput formats for global SH2 profiling based on protein arrays or multiplexed analysis must be established.

In principle, two different formats can be applied to study global SH2 binding profiles with multiple SH2 domains (Fig. 7). The first approach is based on the conventional protein array format. A large panel of different SH2 domains is spotted on the surface of an array, and SH2 binding profiles are quantitatively determined after the binding of labeled sample proteins (Fig. 7A). Several recent publications demonstrated that protein-protein interactions or protein expression profiles can effectively be analyzed with high throughput using different microarray-based formats (92, 93). In these investigations, different antibodies, peptides, kinases, or a large panel of recombinant yeast proteins (representing almost 80% of the yeast proteome) were spotted on the surface of microarrays. Incubation of arrays was performed with single binding proteins, an artificial mixture of different target proteins, or complex mixtures of proteins derived from tissue culture supernatants or whole cellular lysates (94). Bound analytes were detected either by direct labeling of proteins prior to incubation (e.g. single or dual labeling by fluorescent dyes), by target-specific secondary antibodies, or by measuring the enzymatic activity of kinases subsequent to the binding reaction (92, 93, 95).

The reported sensitivity of microarray-based assays is variable, with detection ranging from picograms to micrograms of analyte per milliliter, and is largely dependent on the labeling and detection system used (93). Compared with direct labeling and detection (e.g. by fluorescence), significant improvement in sensitivity was obtained by signal amplification, for example use of biotinylated secondary antibodies and streptavidin-HRP for detection. Limits of detection in the low picogram range were recently reported for a microarray-based sandwich immunoassay in which high sensitivity was achieved by oligonucleotide-labeled antibodies and subsequent signal amplification by rolling circle amplification, allowing the quantification of more than 50 different cytokines in a single reaction (96). In addition to the labeling and detection methods used, the sensitivity of array formats is also dependent on the density and binding characteristics of the immobilized antibodies or proteins, the spotting technology and array surface used, blocking conditions, as well as the complexity of the sample analyzed (97).
SH2 Arrays

In contrast to antibody-based or other protein arrays, there is very little data on comparable array formats for immobilized SH2 domains. In pilot experiments, we tested two different formats for profiling the binding of tyrosine-phosphorylated proteins to immobilized SH2 domains. As a model system, whole cellular lysates from 3T3 fibroblasts and Abl-transformed 3T3 cells were used; the Abl-transformed cells have a much higher level of tyrosine phosphorylation due to the constitutive kinase activity of the Abl oncoprotein (Fig. 8A). In the first approach, different SH2 domains (generated as GST fusion proteins) were immobilized on GSH-derivatized nitrocellulose membranes, and binding reactions were performed with 32P-labeled whole cellular extracts. Cell proteins were labeled in vivo with 32P, and lysed, free 32P, was removed by gel filtration, and blocked membranes were incubated with whole cellular extracts (10 mg/ml, 10^7 cpm) in the presence of 1% BSA for 1 h at room temperature. After extensive washing in Tris-buffered saline/Tween-20, signals were detected by autoradiography. SH2 domains of Nck, Grb2, GAP, and Crk were generated as GST-fusion proteins, and glutathione-derivatized nitrocellulose was used to achieve oriented immobilization of the domains. Immobilized GST served as a negative control. In the second approach, pTyr binding profiles were examined by SELDI-TOF after immobilization of different SH2 domains on protein chip arrays and incubation with whole cellular lysates (data not shown). In contrast to anti-pTyr blots and far-Western blot analyses, we found that no specific pTyr binding profile data could be detected using either format under a variety of assay conditions tested (Fig. 8 and data not shown).

The inability to detect specific binding profiles can be explained either by the loss of binding activity of the SH2 domains due to immobilization or inadequate assay sensitivity and/or poor signal-to-noise ratio. In contrast to the results obtained with whole cellular lysates, control experiments with selected tyrosine-phosphorylated peptides revealed that the immobilized SH2 domains were capable of specific binding to appropriate phosphorylated targets (data not shown). Recently, Espejo et al. reported that labeled peptides or single proteins are efficiently detected by modular binding domains (including SH2 domains) when immobilized on the surface of 3R. Fukunaga and T. Hunter, personal communication.

FIG. 8. A, differences of tyrosine phosphorylation in whole cellular lysates of 3T3 and v-Abl-transformed 3T3 cells. For Western blot analysis, whole cellular lysates were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and tyrosine phosphorylation was detected by anti-pTyr. In the second approach, pTyr binding profiles were examined by SELDI-TOF after immobilization of different SH2 domains on protein chip arrays and incubation with whole cellular lysates (data not shown). In contrast to anti-pTyr blots and far-Western blot analyses, we found that no specific pTyr binding profile data could be detected using either format under a variety of assay conditions tested (Fig. 8 and data not shown).

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This and our data indicate that the binding capability of SH2 domains is not abrogated due to immobilization and point instead to insufficient sensitivity.

In general, signaling proteins such as tyrosine kinase receptors are expressed at low levels of several hundred or thousand molecules per cell. Assuming that a crude protein extract from $10^6$ cells is applied to an array in a volume of 1 ml, limits of detection of between 1 pg/ml and 100 pg/ml are theoretically required for the detection of these low-abundance proteins. As outlined above, comparable sensitivities have been achieved by different antibody-based microarray formats, suggesting that low-abundance proteins such as phosphorylated signaling proteins might be detectable by SH2-based microarray formats. The discrepancy between our observations and antibody-based microarrays may be explained by differences in the binding affinities of antibodies and SH2 domains. While the $K_d$ for antigen-antibody reactions is typically below 1 nM, interactions of SH2 domains with tyrosine-phosphorylated proteins are significantly weaker, with affinities in the range of 100 nM to 10 M (15, 86). Specific binding is further compromised by poor kinetics. Because the rate of binding is proportional to the on-rate and the concentration of analyte, relatively modest on-rates and low analyte concentrations necessarily imply that specific binding will be relatively slow, while nonspecific binding may be more rapid.

Thus, for binding to SH2 arrays, both more sensitive labeling and detection methods and increasing the concentration of the analyte are likely to be required for acceptable signal strength and signal-to-noise ratio. Enrichment of tyrosine-phosphorylated proteins prior to the binding reaction may be achieved by methods already discussed, for example IMAC or anti-pTyr IP (47). However, enrichment can be technically demanding when only small starting amounts of protein are available, and the differences in the relative efficiency of enrichment of different phosphoproteins may bias results. Sensitivity and specificity might also be improved by analyzing different fractions of cellular proteins after chromatographic separation, e.g. by ion exchange or reverse-phase chromatography, but such an approach limits potential throughput (99).

The sensitivity of SH2-based arrays may also be affected by nonspecific binding. Compared with highly sensitive antibody-based sandwich assays in which the analyte is captured by immobilized antibodies and specifically detected by secondary antibodies recognizing the analyte, the binding partners for immobilized SH2 domains in whole cellular extracts are a priori unknown (although a sandwich assay format with anti-pTyr as a detecting antibody is a promising approach to explore (100)). Therefore, SH2-based array analyses require that the protein or peptide sample be unselectively labeled, with the disadvantage of increased background and low signal-to-noise ratios. Moreover, direct labeling is commonly achieved by chemical coupling of labels to peptide reactive groups (e.g. to amino groups by N-hydroxysuccinimide esters); coupling can lead to the inactivation of binding sites and/or decreased solubility, and quantitation may be biased by disproportionate labeling depending on the number of reactive groups present in a given peptide. Taking together current data and theoretical considerations, it remains to be established whether SH2 domain-based array formats are capable of profiling the cellular phosphorylation state of complex protein mixtures.

**Reverse-phase Arrays**

A second strategy for profiling the global cellular SH2-binding state is based on “reverse-phase” formats, in which phosphoproteins present in immobilized cellular lysates are detected by soluble SH2 domain probes (Fig. 7B). The feasibility of this approach is clearly validated by the success of the 1D far-Western approach described above. For immobilization, protein samples are spotted on solid surfaces (membranes, plastic, or glass) or are covalently attached to beads either in their native or denatured state (92, 101). A reverse-
phase microarray approach was recently applied for comparative expression analysis of several prosurvival proteins in microdissected normal and cancerous prostate specimens (65). Levels of protein expression were determined by antibodies, and signal amplification was achieved with biotinylated secondary antibodies in combination with the streptavidin-peroxidase complex. For single immobilized recombinant proteins, the limit of detection was ~1 ng/ml and specific signals were detectable in whole cellular extracts derived from less than 200 cells; intra- and interassay reproducibility varied between 2 and 40% and increased with decreasing amounts of protein immobilized (65).

In pilot experiments, we tested the reverse-phase approach for SH2 profiling in slot-blot or dot-blot formats using immobilized protein extracts of normal and v-Abl-transformed cells (Fig. 9 and data not shown). In accordance with previous far-Western blot analyses, differential binding of individual labeled SH2 domains to whole cellular lysates was reproducibly detectable. To this point, pTyr-specific signals were detected from as little as 0.5 μg of whole cellular lysate, equivalent to ~2500 cells. Although not yet tested on microarray formats, our data suggest that quantitative SH2 profiling could be performed on miniaturized reverse-phase arrays with relatively low amounts of immobilized protein. However, it is likely that for the detection of low-abundance proteins in extracts of small numbers of cells, more sensitive detection methods will be required.

**Toward Higher Throughput**

The reverse-phase approach described above can simultaneously quantitate for many different samples the relative level of binding for a given SH2 domain probe; however, each different probe requires a separate binding reaction. Therefore, to globally profile the SH2 binding state, a large set of identical arrays would be needed, lowering throughput and raising concerns with cost, sample availability, and reproducibility. To overcome this limitation, it would be preferable to measure the binding of multiple SH2 domains in a single reaction; this would be possible only if each SH2 domain probe could be uniquely labeled and the binding of each quantitated simultaneously. Although significant progress has recently been made in the development of different fluorescent dyes, the number of fluorophores with distinct emission spectra is likely to be inadequate for the differential labeling and detection of large numbers of SH2 domains (102).

Differential labeling of SH2 domains with distinct oligonucleotide reporter molecules (DNA tags) is a promising approach to this problem (Fig. 10). For oligonucleotide-based multiplexing, different SH2 domains are labeled with individual DNA tags prior to the binding reaction. The DNA tags are composed of a unique recognition sequence flanked by primer-binding sites for subsequent amplification by polymerase chain reaction (PCR). After binding of the DNA-labeled SH2 domains to immobilized proteins and washing, bound DNA tags are linearly amplified by PCR and subsequently quantitated, for example using PCR-enzyme-linked immunosspot assay, real-time PCR, or oligonucleotide array formats with DNA probes complementary to the individual recognition sequences. Assuming an internal recognition sequence 8 base pairs in length that varies at two nucleotides for different DNA tags, more than 15,000 different combinations can theoretically be discriminated by differential hybridization, easily allowing the differential labeling of the entire panel of SH2 domains required for global profiling.

The concept of DNA labeling of protein probes combined with the power of PCR amplification was originally used for...
the highly sensitive detection of analytes by immuno-PCR (103). In this approach, DNA reporter molecules are coupled to antibodies, allowing the detection of a few hundred protein molecules subsequent to antibody binding and PCR amplification. DNA-antibody conjugates were generated either by direct chemical coupling of antibodies to oligonucleotides or by the formation of complexes between streptavidin, biotinylated oligonucleotides, and antibodies (104, 105). The same strategy can be applied for the differential labeling of SH2 domains. To prevent inactivation or inappropriate biotinylation of the SH2 domains by direct chemical labeling, we applied the biotin protein ligase (BirA) system for biotinylation (106). In this system, the peptide recognition sequence of the BirA enzyme is fused to the C terminus of SH2 domain probes, allowing the controlled enzymatic biotinylation of a single defined residue in the recognition sequence (Figs. 9 and 10). Similar SH2 binding profiles were observed with biotinylated probes complexed with streptavidin-HRP for detection compared with unbiotinylated probes detected with GSH-HRP, indicating that the binding properties of the domains are not affected by the attachment of the peptide recognition sequence and subsequent biotinylation. As only a single defined residue is biotinylated in each probe by the BirA system, disproportionate labeling of proteins and biased quantitation associated with chemical labeling is avoided.

Studies are underway to test oligonucleotide-based multiplexing for quantitative SH2 profiling of complex protein mixtures. Compared with other assay formats for SH2 profiling, oligonucleotide-based multiplexing in combination with the power of PCR amplification is very promising, with the potential to determine the signaling state of a few cells with high sensitivity. It raises the possibility that the binding of hundreds of protein probes, including a comprehensive panel of all SH2 and PTB domains, could be rapidly and simultaneously determined for a single sample. The output from such analyses would be quantitative binding values for each probe, akin to cDNA expression profile data; such quantitative binding information is easily stored in databases and could be analyzed by existing software developed for cDNA expression applications. Because protein tyrosine phosphorylation is arguably much more closely linked to biologically interesting and clinically relevant phenomena than mRNA abundance, such global SH2 binding data are likely to be highly informative and useful.

CONCLUDING REMARKS

The comprehensive profiling of tyrosine-phosphorylated proteins in the cell is an important goal for proteomics; it is likely to reveal a great deal about signaling mechanisms, may serve as a functionally relevant means of classifying cells such as tumors, and may identify important new targets for drug discovery. MS-based phosphoproteomic methods are being developed to address this problem, but issues of sensitivity and throughput remain. SH2 profiling is a sensitive and reasonably rapid method for obtaining a fingerprint of the overall pattern of tyrosine phosphorylation, which is useful in characterizing qualitative and quantitative differences in tyrosine phosphorylation patterns between samples. Further developments of this method will increase sensitivity and allow its application to small amounts of sample or archived clinical samples. The next goal for SH2 binding assays will be the development of multiplexed assays allowing the rapid and simultaneous quantitation of the binding of large numbers of SH2 domains and other protein binding domains to a protein sample. Thus, SH2 binding methods can serve as a valuable complement to large-scale proteomic analyses, with the potential to provide a unique insight into the signaling state of the cell for both basic research and clinical applications.

Acknowledgments—We thank T. Dawson and P. Nash for the list of human SH2 domains, K. Anderson and B. Lin for MM cell lines, J. Fletcher for GIST samples, M. Lalande and M. Landers for p19 cells, Z. Li, R. Bona, N. Patel, J. Kulk, L. Glynn, and E. Laska for clinical specimens, K. Claffey, N. Ryan, and I. Nurieyeva for development of SH2 tissue staining methods, A. Maier for generation of multimerized SH2 probes, R. Fukunaga and T. Hunter for protocols for GSH derivatization of membranes, and C. Thompson and A. Voigt for technical assistance. We are particularly grateful to D. Han and G. Rivera for critical reading of this manuscript.

* This work was partially supported by grants from the National Institutes of Health (CA094378) and the University of Connecticut Health Center, Center for Interdisciplinary Research on Women’s Health (to B. J. M), the Erich and Gertrud Roggenbuck-Stiftung, Hamburg, Germany (to P. N.), and by a General Clinical Research Center Grant to the University of Connecticut Health Center (MO1RR06192) from the National Institutes of Health/National Center for Research Resources.

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REFERENCES

1. Hunter, T. (1989) Protein modification: phosphorylation on tyrosine residues. Curr. Opin. Cell Biol. 1, 1168–1181
2. Kolibaba, K. S., and Druker, B. J. (1997) Protein tyrosine kinases and cancer. Biochim. Biophys. Acta. 1333, F217–248
3. Druker, B. J. (2002) Perspectives on the development of a molecularly targeted agent. Cancer Cell 1, 31–36
4. Montemurro, F., Choa, G., Faggiano, R., Sperti, E., Capaldi, A., Donadio, M., Minischetti, M., Salomone, A., Vitt-Ramus, G., Alabiso, O., and Aglietta, M. (2003) Safety and activity of docetaxel and trastuzumab in HER2 overexpressing metastatic breast cancer: a pilot phase II study. Am. J. Clin. Oncol. 26, 95–97
5. Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) The protein kinase complement of the human genome. Science 298, 1912–1934
6. Adam, G. C., Sorensen, E. J., and Cravatt, B. F. (2002) Chemical strategies for functional proteomics. Mol. Cell. Proteomics. 1, 828–835
7. Lo, L. C., Pang, T. L., Kuo, C. H., Chiang, Y. L., Wang, H. Y., and Lin, J. J. (2002) Design and synthesis of class-selective activity probes for protein tyrosine phosphatases. J. Proteome Res. 1, 35–40
8. Glennen, J. R., Jr., and Zokas, L. (1989) Novel tyrosine kinase substrates
from Rous sarcoma virus-transformed cells are present in the membrane skeleton. J. Cell Biol. 108, 2401–2408

9. Sakai, R., Iwamatsu, A., Hirano, N., Ogawa, S., Tanaka, T., Mano, H., Yazaki, Y., and Hirai, H. (1994) A novel signaling molecule, p130, forms stable complexes in vivo with v-Scr and v-Src in a tyrosine phosphorylation-dependent manner. EMBO J. 13, 3748–3756

10. Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B., and Parsons, J. T. (1992) pp125FAK: a structurally distinct protein-tyrosine kinase associated with focal adhesions. Proc. Natl. Acad. Sci. U. S. A. 89, 5192–5196

11. Panettieri, T. S. (2001) Tyrosine phosphorylation of paxillin, FAK, and p130CAS: effects on cell spreading and migration. Front. Biosci. 7, 143–150

12. Turner, C. E. (2000) Paxillin and focal adhesion signaling. Nat. Cell Biol. 2, E231–E236

13. Zachary, I. (1997) Focal adhesion kinase. Int. J. Biochem. Cell Biol. 29, 929–934

14. Nollau, P., and Mayer, B. J. (2001) Profiling the global tyrosine phosphorylation state by Src homology 2 domain binding. Proc. Natl. Acad. Sci. U. S. A. 98, 13531–13536

15. Bradshaw, J. M., and Wakaman, G. (2002) Molecular recognition by SH2 domains. Adv. Protein Chem. 61, 161–210

16. Watson, P., Gish, D., and Nash, P. (2001) SH2 domains, interaction modules and cellular wiring. Trends Cell Biol. 11, 504–511

17. Adam, G. C., Sorensen, E. J., and Cravatt, B. F. (2002) Chemical strategies for functional proteomics. Mol. Cell Proteomics 1, 781–790

18. Conrads, T. P., Issaq, H. J., and Veenstra, T. D. (2002) New tools for quantitative phosphoproteome analysis. Biochem. Biophys. Res. Commun. 290, 885–890

19. Mann, M., Ong, S. E., Gronborg, M., Steen, H., Jensen, O. N., and Pandey, A. (2002) Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. Trends Biotechnol. 20, 261–268

20. Pandey, A., Andersen, J. S., and Mann, M. (2000) Use of mass spectrometry to study signaling pathways. Sci. STKE 2000, L1

21. Lewis, T. S., Hunt, J. B., Aveline, L. D., Jonascher, K. R., Louie, D. F., Yeh, J. M., Natherne, T. S., Resing, K. A., and Ahn, N. G. (2000) Identification of novel MAP kinase pathway signaling targets by functional proteomics. Mol. Cell 6, 1343–1345

22. Iwafune, Y., Kawasaki, H., and Hirano, H. (2002) Electrophoretic analysis of phosphorylation of the yeast 205kDa proteosome. Electrophoresis 23, 329–338

23. Immler, D., Gremm, D., Kirsch, D., Spengler, B., and Meyer, H. E. (1998) Identification of phosphorylated proteins from thrombin-activated human platelets isolated by two-dimensional gel electrophoresis by electrospary ionization-tandem mass spectrometry (ESI-MS/MS) and liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). J. Am. Soc. Mass Spectrom. 9, 274–288

24. Laron, M. R., Sorensen, G. L., Fey, S. J., Larsen, P. M., and Roepstorff, P. (2001) Phospho-proteomics: evaluation of the use of enzymatic dephosphorylation and differential mass spectrometric peptide mass mapping for site specific phosphorylation assignment in proteins separated by gel electrophoresis. Proteomics 1, 223–238

25. Yoshimura, Y., Shinkawa, T., Taoka, M., Kobayashi, K., Isobe, T., and Yamamura, T. (2002) Identification of protein substrates of Ca2+/calmodulin-dependent protein kinase II in the postsynaptic density by protein sequence and mass spectrometry. Biochem. Biophys. Res. Commun. 290, 948–954

26. Mason, G. G., Murray, R. Z., Pappin, D., and Rivett, A. J. (1998) Phosphoproteome analysis of human sperm. Evidence of tyrosine phosphorylation of cyclin D2 in MCF-7 human breast cancer cells. FEBS Lett. 470, 209–215

27. Ficarro, S. B., McCleland, M. L., Stukenberg, P. T., Burke, D. J., Ross, J. A., Pines, J., and看不到完整的文档内容。
phosphopeptides affinity-bound to immobilized metal ion affinity chromatography beads. Anal. Chem. 74, 3429–3433
47. Ahn, N. G., and Resing, K. A. (2001) Toward the phosphoproteome. Nat. Biotechnol. 19, 317–318
48. Stensballe, A., Andersen, S., and Jensen, O. N. (2001) Characterization of phosphoproteins from electrophoretic gels by nanoscale Fe(III) affinity chromatography with off-line mass spectrometry analysis. Proteomics 1, 207–222
49. Oda, Y., Nagasu, T., and Chait, B. T. (2001) Enrichment analysis of phosphorylated proteins as a tool for probing the phosphoproteome. Nat. Biotechnol. 19, 379–382
50. Zhou, H., Watts, J. D., and Aebersold, R. (2001) A systematic approach to the analysis of protein phosphorylation. Nat. Biotechnol. 19, 375–378
51. Goshe, M. B., Conrads, T. P., Panisko, E. A., Angell, N. H., Veenstra, T. D., and Smith, R. D. (2001) Phosphoprotein isoform-coded affinity tag approach for isolating and quantitating phosphopeptides in proteome-wide analyses. Anal. Chem. 73, 2578–2586
52. Weckwerth, W., Willmitzer, L., and Fiehn, O. (2000) Comparative quantitation of protein expression and site-specific phosphorylation and identification of phosphoproteins using stable isotope labeling and liquid chromatography/mass spectrometry. Rapid Commun. Mass Spectrom. 14, 1677–1681
53. Oda, Y., Huang, K., Cross, F. R., Cowburn, D., and Chait, B. T. (1999) Accurate quantitation of protein expression and site-specific phosphorylation. Proc. Natl. Acad. Sci. U. S. A. 96, 6591–6596
54. Zhang, X., Jin, Q. K., Carr, S. A., and Annan, R. S. (2002) N-Terminal peptide labeling strategy for incorporation of isotopic tags: a method for the determination of site-specific absolute phosphorylation stoichiometry. Rapid Commun. Mass Spectrom. 16, 2325–2332
55. Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999) Quantitative analysis of complex protein mixtures using iso- tope-coded affinity tags. Nat. Biotechnol. 17, 994–999
56. Pande, A., Podtelezhnikov, A. V., Blagoev, B., Bustelo, X. R., Mann, M., and Lodish, H. F. (2000) Analysis of receptor signaling pathways by mass spectrometry: identification of vav-2 as a substrate of the epidermal and platelet-derived growth factor receptors. Proc. Natl. Acad. Sci. U. S. A. 97, 179–184
57. Steen, H., Kuster, B., and Mann, M. (2001) Quadrupole time-of-flight versus triple-quadrupole mass spectrometry for the determination of phosphopeptides by precursor ion scanning. J. Mass Spectrom. 36, 782–790
58. Steen, H., Pande, A., Andersen, J. S., and Mann, M. (2002) Analysis of tyrosine phosphorylation sites in signaling molecules by a phosphotyrosine-specific immunoion screening method. Sci. STKE 2002, L16
59. Saavedra, B. R., Ferreira, V. B., Benevides, C. M., and Pinto, D. S. (2002) ELISA for tyrosine phosphorylation analysis. J. Mass Spectrom. 37, 17–24
60. Steen, H., Andersen, J. S., and Mann, M. (2001) Quadrupole time-of-flight mass spectrometry for the detection of phosphopeptides by precursor ion scanning. J. Mass Spectrom. 36, 782–790
61. Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat. Biotechnol. 17, 994–999
62. Pande, A., Podtelezhnikov, A. V., Blagoev, B., Bustelo, X. R., Mann, M., and Lodish, H. F. (2000) Analysis of receptor signaling pathways by mass spectrometry: identification of vav-2 as a substrate of the epidermal and platelet-derived growth factor receptors. Proc. Natl. Acad. Sci. U. S. A. 97, 179–184
63. Steen, H., Kuster, B., and Mann, M. (2001) Quadrupole time-of-flight versus triple-quadrupole mass spectrometry for the determination of phosphopeptides by precursor ion scanning. J. Mass Spectrom. 36, 782–790
64. Adam, G. C., Cravatt, B. F., and Sorensen, E. J. (2001) Profiling the specific reactivity of the proteome with non-directed activity-based probes. Chem. Biol. 8, 81–95
65. Hondermarck, H., Vercoutter-Edouart, A. S., Revillion, F., Lemoine, J., el-Yazidi-Belkoura, I., Nurcombe, V., and Peyrat, J. P. (2001) Proteomics of breast cancer for marker discovery and signal pathway profiling. Proteomics 1, 1216–1232
66. Issaq, H. J., Veenstra, T. D., Conrads, T. P., and Felschow, D. (2002) The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification. Biochem. Biophys. Res. Commun. 292, 587–592
67. Pawelec, O. P., Charboneau, L., Bichsel, V. E., Simone, N. L., Chen, T., Gienger, J., and Lierse, M. (2001) Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature 415, 141–147
68. Ho, Y., Gruhler, A., Heilbut, A., Bader, G. D., Moore, L., Adams, S. L., Millar, A., Taylor, P., Bennett, K., Boutiller, K. L., Y., and Wolting, C.
90. Schaefer, L. K., Ren, Z., Fuller, G. N., and Schaefer, T. S. (2002) Constitutive activation of Stat3alpha in brain tumors: localization to tumor endothelial cells and activation by the endothelial tyrosine kinase receptor (VEGFR-2). Mol. Cell. Biol. 22, 239–246

94. Knezevic, V., Leethanakul, C., Bichsel, V. E., Worth, J. M., Prabhu, V. V., Gutkind, J. S., Liotta, L. A., Munson, P. J., Petricoin, E. F., 3rd, and Kizzm, D. B. (2001) Proteomic profiling of the cancer microenvironment by antibody arrays. Proteomics 1, 1271–1278

95. Mitchell, P. (2002) A perspective on protein microarrays. Nat. Biotechnol. 20, 225–229

96. Schweitzer, B., Roberts, S., Grimwade, B., Shao, W., Wang, M., Fu, Q., Shu, Q., Laroche, I., Zhou, Z., Tchernev, V. T., Christiansen, J., Veleca, M., and Kingsmore, S. F. (2002) Multiplexed protein profiling on microarrays by rolling-circle amplification. Nat. Biotechnol. 20, 359–365

97. Haab, B. B., Dunham, M. J., and Brown, P. O. (2001) Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. Genome Biol. 2, 4.1–4.13

98. Espejo, A., Cote, J., Bednarek, A., Richard, S., and Bedford, M. T. (2002) A protein-domain microarray identifies novel protein-protein interactions. Biochem. J. 367, 697–702

99. Madoz-Gurpide, J., Wang, H., Misek, D. E., Brichory, F., and Hanash, S. M. (2001) Protein based microarrays: a tool for probing the proteome of cancer cells and tissues. Proteomics 1, 1279–1287

100. Heinrich, M. C., Corless, C. L., Duensing, A., McGreevey, L., Chen, C. J., Joseph, N., Singer, S., Griffith, D. J., Haley, A., Town, A., Demetri, G. D., Fletcher, C. D., and Fletcher, J. A. (2003) PDGFRA activating mutations in gastrointestinal stromal tumors. Science 299, 708–710

101. Petricoin, E. F., Zoon, K. C., Kohn, E. C., Barrett, J. C., and Liotta, L. A. (2002) Clinical proteomics: translating benchside promise into bedside reality. Nat. Rev. Drug Discov. 1, 683–695

102. Tong, A. K., Li, Z., Jones, G. S., Russo, J. J., and Ju, J. (2001) Combinatorial fluorescence energy transfer tags for multiplex biological assays. Nat. Biotechnol. 19, 756–759

103. Sano, T., Smith, C. L., and Cantor, C. R. (1992) Immuno-PCR: very sensitive antigen detection by means of specific antibody-DNA conjugates. Science 258, 120–122

104. Hendrickson, E. R., Truby, T. M., Joerger, R. D., Majarian, W. R., and Ebersole, R. C. (1995) High sensitivity multianalyte immunoassay using covalent DNA-labeled antibodies and polymerase chain reaction. Nucleic Acids Res. 23, 522–529

105. Joerger, R. D., Truby, T. M., Hendrickson, E. R., Young, R. M., and Ebersole, R. C. (1999) Analyte detection with DNA-labeled antibodies and polymerase chain reaction. Clin. Chem. 41, 1371–1377

106. O’Callaghan, C. A., Byford, M. F., Wyer, J. R., Willcox, B. E., Jakobsen, B. K., McMichael, A. J., and Bell, J. I. (1999) BirA enzyme: production and application in the study of membrane receptor-ligand interactions by site-specific biotinylation. Anal. Biochem. 266, 9–15