A Prototype Antibody Microarray Platform to Monitor Changes in Protein Tyrosine Phosphorylation*

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Reversible protein phosphorylation is a key regulatory process in all living cells. Deregulation of modification control mechanisms, especially in the case of tyrosine, may lead to malignant transformation and disease. Phosphotyrosine (p-Tyr) accounts for only 0.05% of the total cellular phospho-amino acid content, yet plays an unusually prominent role in eukaryotic signaling, development, and growth. Tracking temporal and positional p-Tyr changes across the cellular proteome, i.e. tyrosine phosphoproteomics, is therefore tremendously valuable. Here, we describe and evaluate a prototype antibody (Ab) microarray platform to monitor changes in protein Tyr phosphorylation. Availability permitting, a virtually unlimited number of Abs, each recognizing a specific cellular protein, may be arrayed on a chip, incubated with total cell or tissue extracts or with biological fluids, and then probed with a fluorescently labeled p-Tyr-specific monoclonal Ab, PY-KD1, specifically generated for this assay as part of the current study. The optimized protocol allowed detection of changes in the Tyr phosphorylation state of selected proteins using submicrometer to low nanogram of total protein extract, amounts that may conceivably be obtained from a thousand to a hundred thousand cells, or less, depending on the cell or tissue type. The assay platform was evaluated by assessing changes in a rationally selected subset of the Tyr phosphoproteome of Bcr-Abl-expressing cells treated with a specific inhibitor, Gleevec, and of epidermal growth factor (EGF)-treated HeLa cells. The results, ratiometric rather than strictly quantitative in nature, conformed with previous identifications of several Bcr-Abl and EGF receptor targets, and associated proteins, as detected by exhaustive mass spectrometric analyses. The Ab microarray method described here offers advantages of low sample and reagent consumption, scalability, detection multiplexing, and potential compatibility with microfluidic devices and automation. The system may hold particular promise for dissecting signaling pathways, molecular classification of tumors, and profiling of novel target-cancer drugs.

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The complex network of all proteins in a living cell that are stably or transiently phosphorylated at tyrosine residues constitutes the tyrosine phosphoproteome. Reversible tyrosine phosphorylation, through the actions of specialized enzymes, kinases, and phosphatases, plays an important role in cell signaling, development, proliferation, and growth control (1–3). Deregulation has been implicated in disease, most frequently in cancer (4–6). Mutations and other genomic abnormalities such as translocations are known to occasionally cause aberrant activation of selected tyrosine kinases, effecting an unusual phosphorylation state of downstream targets that, in turn, may cause malignant transformation (4, 5, 7). By identifying these targets, diagnostic tools may be developed for some cancers and, importantly, the modification process could be a target for therapeutic intervention (8–11). Monitoring changes in the tyrosine phosphoproteome that accompany cell growth, differentiation, genetic alteration, disease, or exposure to bioactive substances and drugs represents therefore a fundamental task in contemporary proteomics.

Tyrosine phosphorylation is the least abundant post-translational modification (PTM) compared with phospho-serine (p-Ser) or -threonine (p-Thr) and is estimated to be less than 0.05% of the total cellular protein phospho-amino acid content (12). This situation presents a major challenge to develop and implement adequate tyrosine phosphoproteome analysis tools. Mass spectrometry, already the standard method to identify proteins (13), has also gained popularity for phosphoproteome analysis, as practiced either with or without prior gel fractionation of the cellular proteome or subsets thereof (14–20). To this end, trace enrichment procedures have been developed to ensure adequate analysis of phosphotyrosine (p-Tyr) containing proteins, including immunoaffinity-based methods (1, 14, 15, 20, 21), chemical modification of the phosphate moiety for subsequent affinity capture (22, 23),

The abbreviations used are: PTM, post-translational modification; Ab, antibody; mAb, monoclonal antibody; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; p-Tyr, phosphotyrosine; p-Ser, phosphoserine; p-Thr, phosphothreonine; PMF, peptide mass fingerprinting; EDC, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride; mKLH, mariculture keyhole limpet hemocyanin; FPA, forward phase arrays; RPA, reversed phase arrays; MALDI-reTOF, MALDI reflectron TOF; PBST, PBS containing 0.05% Tween® 20; NR, nonredundant; IP, immunoprecipitation.

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and, at least in case of phosphopeptides, IMAC (19, 24, 25). Several technical and practical problems remain unresolved, however, particularly in the analysis of low-abundance proteins in cell or tissue extracts, which necessitates fairly large amounts of starting material. This may be relatively straightforward in the case of cultured cells but very difficult or simply impossible when dealing with tissues. Clinical samples, such as patient biopsies, are unique and often limited in amount and concentration of the analytes. The number of cells required for mass spectrometric identification, typically $10^8$ to $10^9$ (14, 15, 19), may not always exist within the range achievable for clinical studies. It has been estimated (26) that, whereas a cubic centimeter of tissue contains $\sim 10^9$ cells, those numbers could be less than $10^5$ in a core needle biopsy or cell aspirate. It is therefore of major importance to develop new tyrosine phosphoproteome analysis tools with more stringent requirements in terms of sensitivity and throughput. This can be satisfied by exploring antibody (Ab) microarray technology that utilizes extremely low sample and reagent volumes.

In one of the first comprehensive evaluations of an Ab microarray platform for analysis of proteins in complex mixtures, direct fluorescent labeling of the analytes was used for detection at protein concentrations in the low microgram per milliliter range (27). Since then, several other assays based on protein microarrays have been developed utilizing two major formats: forward phase arrays (FPA) and reversed phase arrays (RPA) (26). FPA assays (typically with multiple Abs printed on the chip to probe a single tissue extract or biological fluid sample at a time) have been applied to detect clinically relevant cytokines (28), bacteria and bacterial toxins (29), potential biomarkers in human serum (30), and protein expression profiling in human oral cavity tissues (31) or cultured cells (32). RPA assays (typically with single antigens or multiple antigen-containing samples printed on the chip for incubation with soluble Abs, either purified or in biological fluids) have been utilized in determination of Ab specificity and cross-reactivity (33, 34), detection of auto-Abs in serum from patients with autoimmune disorders (35), screening of human serum for the presence of allergen-specific IgE (36), and expression analysis of a limited number of proteins in esophageal carcinoma and prostate cancer specimens (37).

Despite its proven potential and diversity of many developed applications (38), Ab microarrays have not been widely used in the analysis of the tyrosine phosphoproteome. To this date, only two assay platforms for investigation of PTMs of proteins have been proposed. Grubb and co-workers (39) described an RPA assay for analysis of relative phosphorylation of six cell-signaling proteins in prostate cancer specimens using sequence-specific Abs against p-Tyr-containing peptides. As only small numbers of cells were required, the assay was successfully coupled with laser capture microdissection of clinical specimens. This assay also offers the advantage of parallel analysis of a large number of clinical samples deposited on the same array. The collection of samples must be readily available at the time of array fabrication, therefore making the assay more applicable to screening archived samples from tissue/tumor banks rather than monitoring effectiveness of therapy or compound profiling in drug discovery. Because the assay requires sequence specific Abs, it is also impossible to detect phosphorylation of more than one protein within a single microarray slide. Nielsen and co-workers (40) utilized an FPA approach to achieve simultaneous detection of two tyrosine-phosphorylated proteins. They developed a micro-sandwich assay by arraying anti-ErbB1 (epidermal growth factor receptor, EGFR) and anti-ErbB2 Abs and detecting the phosphorylation signal with corresponding anti-[p-Tyr$^{1068}$]EGFR and anti-[p-Tyr$^{1248}$]ErbB2 Abs. Increasing the number of arrayed elements in that system will require the use of complicated mixtures of corresponding detection Abs. The high complexity of detection “cocktails” may bring about a much-increased probability of Ab cross-reactivity, higher nonspecific binding and overall background, reduction of signal-to-noise ratios, and an elevated cost of experiments.

In the present study, we describe a novel, high-sensitivity, FPA micro-sandwich assay platform, using a labeled p-Tyr-specific Ab and ratiometric data analysis, that is applicable to monitoring changes in the tyrosine phosphoproteome and conducive to a high degree of multiplexing and improved throughput. The reduction of this platform to practice was initially hindered by the absence of one key reagent, namely an anti-p-Tyr monoclonal antibody (mAb) that would meet the following stringent criteria: i) recognition of p-Tyr in all cellular proteins whenever present, ii) no reactivity toward nonphosphorylated tyrosine, iii) no other moieties as part of proteins including phosphorylated serine and threonine are recognized, iv) recognition is independent of the surrounding amino acid sequence, i.e. the recognition epitope is exclusively limited to p-Tyr, and v) a satisfactory performance in microarray-based assays that requires, for example, rather high concentration of mAb. We report herein development of PY-KD1 mAb that satisfies the above criteria and that is much better suited for this technique than the commercially available Abs.

To evaluate our assay platform, we have used the tyrosine phosphoproteome of RT10+ and HeLa cells as model systems. RT10+ cells were originally established by transfecting human megakaryoblastic leukemia cells with the Bcr-Ablexpressing plasmid pGD210 (41). The Bcr-Abl fusion protein is a constitutively active tyrosine kinase and can be specifically inhibited by the anti-cancer drug STI-571 (Gleevec) (8, 9). To select the majority of Abs for the microarray fabrication, we first performed MALDI-TOF mass spectrometric identifications of p-Tyr-containing proteins in RT10+ cells after prior immunocapture on magnetic particles bearing immobilized PY-KD1 mAb. Fabricated Ab microarrays were evaluated by assessing changes in the tyrosine phosphoproteome of i) RT10+ cells after treatment with Gleevec and also of ii) HeLa
cells after treatment with epidermal growth factor (EGF). Analyses can, in principle, be carried out using 10^3 to 10^5 cells, or less, depending on the cell or tissue type. Our studies confirmed the identification of a number of Bcr-Abl and EGFR targets, and associated proteins that had been previously reported. The sandwich Ab microarray assay described herein may hold particular promise for molecular classification of tumors and for compound profiling in development of novel target-cancer drugs similar to Gleevec (8) or Gefitinib (11).

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-p-Tyr Abs were purchased from the following vendors: 4G10 from Upstate USA, Inc. (Charlottesville, VA), PY-20 from BD Transduction Laboratories (Los Angeles, CA), and PY-100 from Cell Signaling Technology (Beverly, MA). Secondary Abs and streptavidin conjugated to horseradish peroxidase, mariculture keyhole limpet hemocyanin (mKLH), maleimide-activated BSA, D-Salt™ dextran columns, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), Imject® conjugation buffers, ImmunoPure® TMB substrate kit, Hal™ protease inhibitor mixture, micro BCA™ protein assay reagent kit, and Slide-A-Lyzer® dialysis cassettes were obtained from Pierce (Rockford, IL). Recombinant Yersinia protein tyrosine phosphatase was obtained from Calbiochem (San Diego, CA). IgG-free, protease-free BSA was obtained from Jackson Immunoresearch (West Grove, PA). Acetylated p-Tyr was custom synthesized by AnaSpec, Inc. (San Jose, CA) and all peptides by the Microchemistry Core Facility (MSKCC, New York, NY). Protein molecular weight markers were purchased from Amersham Biosciences (Piscataway, NJ), Ready Gel® polyacrylamide precast gels, polyoxyethylene sorbitan monolaurate (Tween® 20), and PVDF membranes were obtained from Bio-Rad Laboratories (Hercules, CA). Other reagents and supplies were obtained from Sigma (St. Louis, MO).

**mAb Development**—p-Tyr was coupled to mKLH using glutaraldehyde as described previously (42). Excess of nonconjugated p-Tyr was removed by passing the reaction mixture through a cross-linked dextran gel filtration column equilibrated with a purification buffer containing mKLH stabilizers (Pierce). Prepared conjugate was aliquoted and stored frozen until used. Immunization of animals, fusion, and purification of the mAb was performed by the Monoclonal Antibody Core Facility (MSKCC), and all animal work was done under the protocol approved by the IACUC (Institutional Animal Care and Use Committee). Briefly, 50 µg of the antigen was emulsified in 500 µl of 50% TiterMax® adjuvant and used for the first intraperitoneal immunization of female BALB/c mice. Three weeks later, mice were injected intraperitoneally with 50 µg of the same conjugate emulsified in TiterMax®; after an additional 3 weeks, the intraperitoneal immunization with 50 µg of antigen emulsified in TiterMax® was repeated. Four weeks later, the last injection of 50 µg of immunogen in PBS was performed intravenously, and after 3 days, the animal was sacrificed, and the spleen was removed and fused with SP2/0-Ag14 myeloma cells (American Type Culture Collection, Manassas, VA). Test bleed was performed 1 week after each injection and before sacrificing the animal (terminal bleed). Fused cells were grown in hybridoma SFM medium supplemented with 10 mM sodium hypoxanthine, 1.6 µM thymidine, 15% fetal bovine serum (v/v) and 1% hybridoma cloning factor (v/v). Hybridoma SFM medium and HT supplement were from Invitrogen (Carlsbad, CA) and Origene® hybridoma cloning factor from Fisher Scientific (Pittsburg, PA). Hybridomas that secrete anti-p-Tyr Abs were selected by ELISAs and sub-cloned three times by the limited dilution method. PY-KD1 mAb was produced in vitro using a CELLine bioreactor (Integra BioSciences, Chur, Switzerland) and purified on a HiTrap™ protein G column (Amersham Biosciences) according to the manufacturer’s recommendations.

**ELISA**—Assays were performed by coating 96-well 4HBX microplates (Dynex Technologies, Chantilly, VA) with 75-ng/well hapten-BSA conjugate in PBS. Conjugated peptides contained either N- or C-terminal cysteine for easy covalent attachment to maleimide-activated BSA. Acetylated p-Tyr was coupled to BSA using EDC. Conjugation reactions were performed as recommended by Pierce. Excess of nonconjugated hapten was removed by dialysis against PBS using dialysis membranes with a molecular weight cut-off 7,000. Prepared conjugates were aliquoted and stored at −20 °C until further use. Coating of microplates was performed overnight at 4 °C. Prepared microplates were washed four times with PBS containing 0.05% Tween® 20 (PBSB), blocked with 10 mg/ml BSA in PBSB for 1 h at 37 °C and washed four times with PBSB again. Samples (sera from immunized mice or hybridoma supernatants) were incubated in microplates for 1 h at 37 °C followed by four washes with PBSB. Horseradish peroxidase-conjugated secondary Ab was added to the plates and incubated for 1 h at 37 °C followed by four washes with PBSB. Color development was achieved with ImmunoPure® TMB substrate reagent kit according to the manufacturer recommendations. Plates were read at 450 nm in a Bio-Rad 550 microplate reader.

**Cell Cultures**—Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. RT10+ cells were obtained from Dr. Bayard Clarkson (MSKCC) and grown in Iscove’s modified Dulbecco’s medium supplemented with 20% (v/v) heat-inactivated fetal bovine serum and a penicillin-streptomycin mixture. They were maintained twice weekly by addition of fresh medium to a dilution of 2 × 10⁵ cells/ml. HeLa cells (American Type Culture Collection) were grown in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and a penicillin-streptomycin mixture. Upon reaching confluence (approximately every 4 days), they were routinely passaged by trypsinization. RT10+ cultures (density 1.2–1.5 × 10⁶ cells/ml) were treated with Gleevec (1 µM) for 2 h. Subconfluent cultures of HeLa cells were grown in serum-free medium for 24 h followed by EGF treatment (150 ng/ml) for 1 h. S15-T71 (Gleevec) was synthesized by the Preparative Chemistry Core Facility (MSKCC); tissue culture grade EGF was purchased from Upstate USA, Inc.

To prepare lysates, cells were collected and washed four times with ice-cold PBS without calcium or magnesium. The extraction buffer typically was Tris-HCl (50 mM, pH 7.3) supplemented with EDTA (1 mM), IGEPAL (1%), NaF (1 mM), Na3VO4 (2 mM) and Hal™ protease inhibitor mixture (1×). Ice-cold extraction buffer was added to cells (1 ml per 10⁷ cells). Proteins were extracted for 15 min on a rocking platform at 4 °C. Cell debris was removed by centrifugation at 15,000 × g for 30 min at 4 °C. Protein concentration of the extract was determined using the Pierce micro BCA reagent kit. When the cell lysate was prepared for subsequent direct labeling with a fluorescent dye, the Tris buffer was substituted by carbonate buffer (200 mM, pH 9.3).

**Protein and Ab Labeling**—In a typical experiment, 2.6 ml of protein extract (0.2 mg/ml) was labeled with Cy5 fluorescent dye (Amersham Biosciences). The dye (200 nmol) was dissolved in a total volume of protein extract to be labeled and incubated in the dark at room temperature and gentle rocking for 30 min. Separation of unincorporated dye was done by gel-filtration over a Sephadex G-25 column (Amersham Biosciences), equilibrated with PBSB. An equal volume of unlabeled protein extract was also applied to a G-25 column to exchange the extraction buffer to PBSB. The same labeling/purification procedure was used to attach Cy5 dye to the PY-KD1 mAb.

Biotinylation of the mAb was performed using EZ-Link™ sulfo-NHS-LC-biotin (Pierce). Two micrograms of purified, anti-p-Tyr IgG1 (clone PY-KD1), in a final volume of 1 ml of PBS, was mixed with 27 µl of 10 mM reagent for 1 h at room temperature on an end-over-end
mixture. Excess free biotin was removed by extensive dialysis against PBS using a dialysis membrane with a 10,000 molecular weight cut-off. Biotinylation was determined to be approximately six molecules of biotin per molecule of mAb by using the EZ®-Tm biotinylation kit (Pierce) according to the manufacturer’s procedure.

**Immunofinity Capture of p-Tyr-Containing Proteins** —Ten milligrams of streptavidin-coated paramagnetic particles (Dynabeads® M-280; Dynal Biotech ASA, Oslo, Norway) were collected, washed with 1 ml of PBS for 10 min, and collected; this cycle was repeated twice. The final pellet was resuspended in the solution of biotinylated PY-KD1 mAb (~4 mg mAb in 2 ml of PBS) and incubated for 3 h at room temperature on an end-over-end mixer. The Ab-coupled particles were then washed for 10 min; twice with PBS, 0.1% Tween® 20; twice with PBS, 0.1% Triton® X-100; and twice with PBS. For collection of the beads from large volumes, high-powered cobalt magnetic discs (catalog no. CR30352-75; Edmund Scientific, Tonawanda, NY) were placed underneath 15-ml Falcon tubes during centrifugation in a GS-6KR rotor (Beckman, Fullerton, CA) at 700 × g for 5 min at 4 °C. For smaller volumes, in Eppendorf tubes, a hand-held MPC-S magnet (Dynal Biotech) was used. mAb beads were stored at 4 °C until further use.

RT10+ cells (5 × 10^6) were washed with ice-cold PBS and resuspended in 50 ml of ice-cold RIPA buffer (50 mM Tris buffer, pH 7.4, 1% IGEPEAL, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM PMSF, 1 mM Na_3VO_4, containing 1× Halt® protease inhibitor and phosphatase inhibitor mixture). Cells were lysed for 15 min on a shaking platform at 4 °C, centrifuged at 14,000 × g for 15 min at 4 °C, and the supernatant stored at −80 °C. Lysate (50 ml, 0.5 mg/ml protein) was incubated with 5 mg of mAb beads overnight at 4 °C on an end-over-end mixer. The particles were pelleted as described above, transferred to a 2-ml tube, and washed for 10 min at 4 °C; thrice with PBS; thrice with PBS, 0.1% Tween® 20; and thrice with PBS, 0.1% Triton® X-100. p-Tyr-containing proteins were eluted in 20 μl of 20 mM phenylphosphate (in PBS) for 2 h at 4 °C under slight agitation. The eluate was then mixed with 20 μl of 2× Laemmli buffer, heated at 60 °C for 10 min, and the proteins were separated on a precast 4–15% Tris-glycine polyacrylamide gel (Bio-Rad) and stained with Coomasie® Brilliant Blue R-250 (Bio-Rad).

**Mass Spectrometry** —Gel-resolved proteins were digested with trypsin, the mixtures fractionated on a Poros 50 R2 RP micro-tip, and resulting peptide pools individually analyzed by MALDI reflectron TOF/TOF (MALDI-TOF/TOF) MS using a Bruker UltraFlex TOF/TOF instrument (Bruker Daltonics, Bremen, Germany), in the presence of three peptide calibrants (6 fmol each; calculated monoisotopic masses of 2,108.155 Da, 1,307.762 Da, and 969.575 Da in the protonated form), as described (43, 44). Spectra were obtained by averaging multiple signals; laser irradiance and number of acquisitions (typically 100–150) were operator-adjusted to yield maximal peak deflections derived from the digitizer in real time. The monoisotopic masses were assigned for all prominent peaks after visual inspection, and the low- and high-end internal standards were used for recalibration. Pass/fail criterion for recalibration is correct assignment of an m/z value for the middle calibrate with a mass accuracy equal or better than 15 ppm. After removal of autolytic and keratin-derived tryptic peptides, selected fragment ions (m/z) were taken to search the human segment database (NC; ~109,000 entries; National Center for Biotechnology Information, Bethesda, MD) utilizing the PeptideSearch algorithm (Matthias Mann, Southern Denmark University, Odense, Denmark; an updated version of this program is currently available as “PepSea” from MDS-Denmark). A molecular mass range double the apparent molecular weight (as estimated from gel electrophoretic relative mobility) was covered, with a mass accuracy restriction better than 40 ppm, and a maximum of one missed cleavage site allowed per peptide. Iterative cycles of database searching and removal of all m/z values that matched a chosen protein sequence were carried out.

To confirm some of the peptide mass fingerprinting (PMF) results, limited MS sequencing of selected peptides was done by MALDI-TOF/TOF (MS/MS) analysis on the same prepared samples, using the UltraFlex instrument in “LIFT” mode. Fragment ion spectra were then taken to search the NR database using the MASCOT MS/MS ion Search program, version 2.0.04 for Windows (Matrix Science Ltd., London, United Kingdom) (45). Any tentative confirmation (Mascot score ≥ 30) of a PMF result thus obtained was further verified by comparing the computer-generated fragment ion series of the predicted tryptic peptide with the experimental MS/MS data. No identifications in this study were based on MS/MS data alone.

**Immunoprecipitation and Western blotting** —Ten micrograms of purified specific Ab was added to 500 μl (0.3–1.2 mg of protein) of cell lysate and incubated at 4 °C for 3 h with slow rotation on a Dynal sample mixer. Dynabeads® Protein G beads (50 μl) were washed four times with PBS using a Dynal M-PCS-S magnet. The beads were then added to the cell lysate-Ab mixture and incubated at 4 °C for 1 h with slow rotation. After completion of the incubation, the beads were washed again as described above, suspended in 50 μl of 1× Laemmli sample buffer, and boiled for 5 min. The beads were pulled down with the magnet, and supernatant was used for analysis. The SDS-gel electrophoresis and Western blotting were performed as described elsewhere (46). The concentration of PY-KD1 or protein-specific Abs used in Western blotting was 1 μg/ml. The concentration of secondary Abs conjugated to horseradish peroxidase was 200 ng/ml. The detection of immune complexes was performed with the ECL™ reagent kit (Amersham Biosciences) according to instructions provided by the manufacturer. The films were scanned and respective bands quantified using a Molecular Dynamics Personal Densitometer SI and ImageQuant software (Amersham Pharmacia Biotech).

**Microarray Fabrication** —Abs for microarray fabrication were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and printed on HydroGel® coated slides (Perkin Elmer Life Sciences, Boston, MA) using MicroSpot 2500 pins and a BioRobotics MicroGrid II arrayer (Genomic Solutions, Ann Arbor, MI). Printing ink was PBS containing 0.2% gelatin and 0.1% sodium azide. Printing concentration of each Ab was 200 μg/ml. Each Ab was spotted onto the array at least nine times. The spacing between spots was 300 μm. Quality control of Ab deposition was performed using a Cy3-labeled nonspecific Ab. Quality control of retention of Abs was performed using deposition of mouse IgG that was detected at the completion of experiments with Cy3-labeled goat anti-mouse Ab. After completion of a printing cycle, arrays were incubated in the dark at room temperature and 65% relative humidity for at least 48 h. They were washed three times with PBST for 30 min on an orbital shaker. Finally, they were dipped in PBS, centrifuged at 100 × g for 5 min, and left at 37 °C for a few minutes to allow to dry completely. Arrays were stored in a noncondensing atmosphere at 4 °C.

Before an experiment, arrays were allowed to reach room temperature and blocked with a PBST solution containing 10 mg/ml BSA for at least an hour with gentle agitation. Arrays were then dipped in PBS, centrifuged at 100 × g for 5 min, and placed at 37 °C for a few minutes to allow drying. Each microarray was incubated with 100 μl of Cy3-labeled protein (1–100 μg/ml) extract for 1 h at 37 °C. Protein extract was supplemented with 1 mg/ml BSA. Upon completion of incubation, arrays were washed four times with PBST for 15 min at room temperature on an orbital shaker. They were dipped in PBS, centrifuged at 100 × g for 5 min, and left at 37 °C for a few minutes to allow drying. Arrays were scanned using a GeneArray microarray scanner (Affymetrix, Santa Clara, CA).
Tyrosine Phosphoproteome Analysis

| Table I |
|---------------------------------|
| **Hapten-BSA conjugates used in Ab screening** |

| Conjugate ID | Amino acid sequence of hapten |
|--------------|------------------------------|
| 1            | Cys-Ile-Glu-Asp-Ala-Ile-pTyr-Ala-Ala-Arg-Gly |
| 2            | Tyr-Ile-pTyr-Gly-Ser-Phe-Lys-Cys |
| 3            | Cys-Pro-Glu-Glu-Thr-Gln-pTyr-Gln-Asp-Gln-Pro-Met-Glu |
| 4            | Cys-Met-Asn-pTyr-Leu-Arg-Arg-Leu-Ser-Asp-Ser-Phe-Glu |
| 5            | Ac-pTyr |
| 6            | Cys-Met-Asn-Tyr-Leu-Arg-Arg-Leu-Ser-Asp-Ser-Phe-Glu |
| 7            | Ac-Tyr-Ahx-Cys |
| 8            | Cys-Ile-Glu-Asp-Ala-Ile-Tyr-Ala-Ala-Arg-Gly |
| 9            | Cys-Pro-Glu-Glu-Thr-Gln-Tyr-Gln-Asp-Gln-Pro-Met-Glu |
| 10           | Cys-Pro-Glu-Glu-pThr-Gln-pThr-Gln-Asp-Gln-Pro-Met-Glu |
| 11           | Cys-Gly-Arg-Ala-Arg-pThr-Ser-Ser-Phe-Ala-Glu |
| 12           | Tyr-Ile-Tyr-Gly-pThr-Phe-Lys-Cys |
| 13           | Cys-Ile-Glu-Asp-Ala-Ile-pThr-Ala-Ala-Arg-Gly |
| 14           | Cys-Pro-Glu-Glu-Thr-Gln-Thr-Gln-Asp-Gln-Pro-Met-Glu |
| 15           | Cys-Gly-Arg-Ala-Arg-Thr-pSer-Ser-Phe-Ala-Glu |
| 16           | Tyr-Ile-Tyr-Gly-pSer-Phe-Lys-Cys |
| 17           | Cys-Ile-Glu-Asp-Ala-Ile-pSer-Ala-Ala-Arg-Gly |
| 18           | Cys-Met-Asn-Tyr-Leu-Arg-Arg-Leu-Ser-Asp-pSer-Phe-Glu |
| 19           | Cys-Gly-Arg-Ala-Arg-Thr-pSer-Ser-Phe-Ala-Glu |
| 20           | Cys-Ile-Glu-Asp-Ala-Ile-Ser-Ala-Ala-Arg-Gly |

Detection of p-Tyr Proteins Using Microarrays—Each microarray was incubated with 100 μl of unlabeled protein extract (0.001–100 μg/ml) for 1 h at 37 °C. Protein extract was always supplemented with 1 mg/ml BSA. Upon completion of incubation, arrays were washed with PBST four times for 15 min at room temperature on an orbital shaker. Cy5-PY-KD1 mAb was diluted in an Ab dilution buffer (PBST with 1 mg/ml BSA) to a final concentration 5 μg/ml, and 100 μl of this solution was incubated with each array for 1 h at 37 °C. Arrays were washed with PBST four times for 15 min at room temperature on an orbital shaker. They were dipped in PBS, centrifuged at 100 g for 5 min, and left at 37 °C for a few minutes to allow them to dry. Arrays were scanned using a GeneArray microarray scanner.

Microarray Data Analysis—The location of each Ab feature on the array was determined by creating a Gal file with the clone tracking option of MicroGrid II software (Genomic Solutions) and importing it to GenePix Pro 4.0 software (Axon Instruments, Union City, CA). The fluorescence signal from each spot was determined as the average of the pixel intensities within the boundary outlined by the software. The local background was subtracted from the signal at each spot. Corrected signal to local background (signal-to-noise) ratio was then calculated for each spot (arbitrary signal). Spots with a net fluorescence below 150 or with obvious defects were eliminated from analysis. Fold reduction of the arbitrary signal by EGF, \( R_{pTyr} \), was calculated as the ratio of arbitrary signals obtained from extracts of untreated and Gleevec-treated RT10+ cells. Fold induction of the arbitrary signal by EGF, \( R_{pTyr} \), was calculated as the ratio of arbitrary signals from extracts of EGF-treated and serum-starved, untreated HeLa cells.

RESULTS

Development of the PY-KD1 mAb—Hapten-BSA conjugates used in the ELISAs to test the polyclonal immune response in mice are shown in Table I. To ensure selection of hybridomas secreting high-affinity Abs to p-Tyr that satisfy the criteria outlined in the introduction, we applied a five-step screening strategy with sequential elimination of negative, low-affinity, cross-reactive, or sequence-dependent clones.

The primary screen was performed against p-Tyr haptens (conjugate 5 and equimolar mixture of conjugates 1, 3, and 4, see Table I). Approximately 7% clonal wells displayed reactivity significantly above control level (medium from fusion wells with no cell growth) and were considered positive. To eliminate clones recognizing nonphosphorylated Tyr and sequence-dependent p-Tyr clones, the secondary screen was performed against Tyr hapten (equimolar mixture of conjugates 8 and 9) and individual p-Tyr hapten (conjugates 1, 3, and 4). The tertiary screen was done against individual conjugates 6, 7, 10, 13, 16, and 18 to confirm absence of reactivity toward nonphosphorylated Tyr and eliminate clones recognizing p-Thr and p-Ser. Multiple subcloning procedure was applied to secure stable hybridomas producing anti-p-Tyr Abs. The quaternary screen was performed with resulting subclones against p-Tyr hapten (individual conjugates 5, 7, and equimolar mixture of conjugates 1, 3, and 4) and Tyr hapten (equimolar mixture of conjugates 6, 8, and 9). The final screen was done against individual conjugates 1, 3, 4, 5, 6, 7, 10, 11, 13, 16, and 18. Based on the final screen results, a stable clone PY-KD1 producing anti-p-Tyr mAb was selected. Isotyping of PY-KD1 mAb demonstrated that it belongs to IgG1 (\( \lambda \)) class.

Comparison of PY-KD1, 4G10, PY-20, and PY-100 mAbs—All Abs were tested by immunoblotting using cell extracts from several cell lines and produced identical banding patterns when used at concentration 100 ng/ml or less (data not shown). Extensive ELISAs were performed using a serial dilution of affinity-purified PY-KD1 mAb and its commercial counterparts 4G10, PY-20, and PY-100. At higher dilutions (0.5–60 ng/ml), all Abs reacted with p-Tyr but not with p-Thr, p-Ser, and nonphosphorylated Tyr (data not shown). How-
ever, important differences were observed in the range of 0.05–6 μg/ml (Fig. 1). Under those conditions, all Abs recognized p-Tyr (conjugates 1, 3, 4, and 5), but the PY-20 mAb also displayed a high degree of reactivity toward nonphosphorylated Tyr (conjugates 6 and 7), p-Thr (conjugates 10, 11, and 13) and p-Ser (conjugates 16 and 18), and PY-100 and 4G10 had measurable reactivities toward nonphosphorylated Tyr (conjugates 6 and 7). No reactivity of PY-KD1 mAb toward p-Thr, p-Ser, or nonphosphorylated Tyr could be measured, not even at the highest reagent concentration (6 μg/ml), which makes it a clear choice for sandwich-based immunodetection of p-Tyr in a microarray format.

**Testing of the PY-KD1 mAb in Microarray Experiments**—A schematic overview of the sandwich-based microarray platform for p-Tyr detection is shown in Fig. 2, and comprises i) immobilization of various commercially available “anti-protein” Abs on a chip, ii) incubation of the chip with a cell or tissue extract, and iii) application of fluorescently labeled detection Ab PY-KD1. To examine the performance of PY-KD1 mAb in this assay format, we fabricated a prototype phosphorylation array by depositing Abs against SHIP (that recognizes both SHIP-1 and SHIP-2), Raf-1, MEK-2, ERK-1, and Dok-2 on HydroGel™-coated slides. The HydroGel™ surface was selected because polyacrylamide provides a high capacity, low nonspecific binding, and low self-fluorescence support for immobilization of Abs on microchips (47). RT10+ cells were then taken for an initial screen as they contain unusually high levels of tyrosine phosphorylation (41, 48) and thus represent an ideal source of protein to probe for these particular modifications. The five targets, all signaling proteins, were chosen to include some of the previously identified Tyr-phosphorylated species (SHIPs and Dok-2) (48), as well as others not (yet) shown to be modified in this manner, in these type of cells. Presence of each cognate protein was positively assessed by Western blot analysis (data not shown).

The arrays were first incubated with fluorescently (Cy5) labeled, total protein extract at concentrations ranging from 1 to 100 μg/ml. A comparable (less than 2-fold variation) fluorescence signal was registered for all five proteins (Fig. 3, upper panel) and the optimum detection concentration was determined to be around 10 μg/ml of total protein incubated with the chip. A different set of arrays was then incubated with unlabeled extract from RT10+ cells at total protein concentrations ranging from 0.001 to 100 μg/ml. Detection of p-Tyr was done with Cy5-labeled PY-KD1 mAb (5 μg/ml) and the optimum total protein concentration was 10 ng/ml, about 1,000-fold lower than for “direct-labeling” detection. This time, clear differences were noted (Fig. 3, lower panel), with the most intense signal (for SHIP) about 5-fold higher than the weakest ones (Raf-1 and MEK-2), which is in good agreement with earlier tyrosine-phosphoproteomic profiling studies of Bcr-Abl-expressing cells using classical biochemical and mass spectrometric means (19, 48). In fact, relative to the arbitrary signals of direct-labeling detection of the cognate
proteins (upper panel), the p-Tyr-derived signal of SHIP was about 10 times that of Raf-1, suggesting sizable differences in the rates of modification. This was by no means the result of an unusually high background signal as can be easily inferred from the sharp contrast and crisp boundaries of even the weaker spots in both high- and low-magnification array images (Fig. 3). As the data was reproducible over at least five independent experiments, we considered the results sufficiently encouraging to test the approach using a larger panel of Abs.

**Protein Immunocapture Using PY-KD1 mAb for Mass Spectrometric Identification**—In designing a custom Ab chip for monitoring changes in tyrosine phosphorylation in RT10+ cells, we wanted to include as many potential p-Tyr-derivatized or -interacting proteins as possible, availability of Abs permitting. A number of putative targets of the oncogenic Bcr-Abl kinase activity have previously been identified in human K562 erythroleukemia cells and mouse myeloid 32Dp210 cells (19). To independently confirm these results and to possibly find more or different targets in the RT10+ megakaryoblastic leukemia cells, purified PY-KD1 mAb was used for solid-phase affinity-capture after prior biotinylation and binding to streptavidin-coated magnetic particles. Total protein
extract from $5 \times 10^9$ cells was then mixed with the derivatized beads and, after extensive washing of the beads, the bound proteins were eluted in a minimal volume of phenyl phosphate-containing buffer, and followed by gel electrophoresis separation and visualization by Coomassie staining. The resulting banding pattern is shown in Fig. 4. Bands were excised, proteins digested with trypsin, and identified, primarily by PMF using MALDI-reTOF MS. In selected cases, the result was confirmed by MALDI-TOF/TOF MS/MS analysis of selected peptides. See “Experimental Procedures” for details. Protein identities and positions on the gel are shown, along with the NCBI GI numbers. Molecular mass markers are indicated on the left.

Tyrosine Phosphoproteome Analysis

Nine (i.e. Bcr-Abl, SHIP-2, Cbl, Syk11, SH-PTP2, DOK-1 and 2, Shc, and E3BP) of those 22 proteins had also been observed by Salomon and coworkers in a related analysis, out of a total 19 proteins identified in two types of cells in that particular study (19). This represents less than 50% overlap between the two datasets. It should be noted that in the earlier study, affinity-captured p-Tyr proteins were digested and the phosphopeptides selectively retrieved by immobilized-metal chromatography before identification by MS/MS. In this way, the chance that any associated, unphosphorylated proteins may have been identified was greatly diminished, which could explain some of the differences, in addition to possible anomalies owing to the use of diverse cell types as the source of protein. Taken together, 32 unique proteins have been retrieved by immunocapture from Bcr-Abl oncogenic fusion-expressing cells, using anti-p-Tyr mAbs, in these two studies combined.

Selection and Characterization of Abs for Microarray Fabrication—Commercially available Abs were obtained for 18 of the 32 potential targets (Table II; 1–18; group “A”); eight of which have been identified in two independent analyses (no. 1, 5, 7, 8, 10–13), eight others in this report (no. 2–4, 6, 9, 14–16) and two more by Salomon et al. (no. 17, 18). We realized that some of these proteins, GRB2 for example, were actually not “direct” targets of the Bcr-Abl kinase activity but rather interacted with p-Tyr proteins. Nevertheless, they will likely produce an “indirect” signal on the array (see Fig. 2; the captured protein binds a p-Tyr protein, which is in turn recognized by the labeled PY-KD1 mAb) and therefore be useful for comparative analysis, even if the bound phospho-protein(s) is (are) unknown. We then also selected 10 Abs for cognate proteins (no. 19–28; group “B”) also previously shown to be tyrosine-phosphorylated, albeit not necessarily in human myeloid leukemia cells. Finally, we included seven Abs, recognizing proteins (no. 29–35; group “C”) that, to the best of our knowledge, have never been shown to contain p-Tyr. All 35 Abs were first characterized in a series of immunocapturing (with PY-KD1 beads) and immunoprecipitation experiments, followed by Western blot analyses using either PY-KD1 mAb or each of the 35 “self” Abs. The results are summarized in Table II.

Twenty-three of the 35 cognate proteins could be captured from RT10+ cell extracts using immobilized PY-KD1 mAb, as indicated by a positive signal in subsequent Western blots with each specific “self” Ab; 14/18 in group A, 7/10 in group B, and 2/7 in group C. It is unlikely that the four group-A proteins (Gads, Shc, Dock180, and Cas-L) not detected in this analysis had been misidentified as Shc was independently identified twice and Gads yielded PMF sequence coverage of ~30% (Table II). Rather, the Abs may not have worked in the Western blot. Of the 14 group-A proteins that were confirmed in the bead pull-downs, 10 (c-Abl, p67phox, PI3Kp85, SHIP-1 and 2, SH-PTP2, DOK-1 and 2, Syk, and Gab1) were found to be bona fide p-Tyr proteins, as species of the correct molec-
ular mass were detected in Western blots with PY-KD1 after immunoprecipitation (IP) with "self" Abs. By contrast, three cognate proteins (GRB2, Cbl, RasGap) in the bead pull-downs were detected after similar IPs when probed with "self" Abs but not with PY-KD1, indicating an apparent absence of p-Tyr. SLP-76 could not be immunoprecipitated; i.e. there was no signal in Western blots using "self" Abs.

Of the seven group-B proteins detected in the bead pull-downs, three (PLC-γ1, Crk-L, and Raf-1) were likely Tyr-phosphorylated; the four others (ZAP-70, IRS-1, ERK-1, and NFκBp65) were not immunoprecipitated with "self" Abs. Interestingly, Lck, which was not detected in the earlier pull-down with PY-KD1, was also found to be Tyr-phosphorylated after IP with an anti-Lck Ab. Lck is a well-characterized p-Tyr protein in T cells (49), and failure to recover it in our bead pull-down could be due to an inaccessible phosphorylation site. As for members of the "negative control" group (C), presence of both cyclin A and MEK-1 was confirmed.

**Tyrosine Phosphoproteome Analysis**

### TABLE II

| No. | Proteina | Identified | p-Tyr proteind | Ab catalogno.e | PY-KD1 capture & WBf | IP with proteinspecific Ab | WB with "self" Abg.h | WB with PY-KD1i |
|-----|----------|------------|----------------|----------------|------------------------|--------------------------|---------------------|-----------------|
|     |          | This studyb | Otherc          |                |                        |                          |                     |                  |
| Group A | 1     | c-Abl     | 14/29; 17.6% x | x              | sc-131      | x x                      | x x                  |                  |
|   | 2     | p67-phox  | 5/12; 11.8% x | x              | sc-7663     | x x                      | x x                  |                  |
|   | 3     | PI3-kinase p85α | 10/19; 11.4% x | x              | sc-423      | x x                      | x x                  |                  |
|   | 4     | SHP-1     | 11/27; 11.0% x | x              | sc-1964     | x x                      | x x                  |                  |
|   | 5     | SHP-2     | 22/36; 21.1% x | x              | sc-14504    | x x                      | x x                  |                  |
|   | 6     | GRB2      | 9/17; 37.3% x | x              | sc-255      | x x                      | x x                  |                  |
|   | 7     | SH-PTP2   | 7/19; 13.3% x | x              | sc-7384     | x x                      | x x                  |                  |
|   | 8     | Dok-1     | 14/26; 40.5% x | x              | sc-6277     | x x                      | x x                  |                  |
|   | 9     | Gads      | 9/14; 30.3% x | x              | sc-12014    | x x                      | x x                  |                  |
|   | 10    | Cbl       | 12/26'; 17.6% x | x              | sc-1651     | x x                      | x x                  |                  |
|   | 11    | Dok-2     | 19/43; 35.9% x | x              | sc-8130     | x x                      | x x                  |                  |
|   | 12    | Syk       | 8/13; 13.9% x | x              | sc-1077     | x x                      | x x                  |                  |
|   | 13    | Shc       | 12/24'; 22.6% x | x              | sc-967      | x x                      | x x                  |                  |
|   | 14    | Ras GAP   | 23/49; 29.6% x | x              | sc-63       | x x                      | x x                  |                  |
|   | 15    | DOCK 180  | 16/43; 10.7% x | x              | sc-6167     | x x                      | x x                  |                  |
|   | 16    | SLP-76    | 6/15; 15.0% x | x              | sc-1961     | x NS NS                  | x NS NS              |                  |
|   | 17    | Gab-1     | x x            | sc-6292      | x x         |                          |                      |                  |
|   | 18    | Cas-L     | x x            | sc-6848      | x x         |                          |                      |                  |
| Group B | 19    | PLC-γ1    | x sc-7290     | x x          | x x         |                          |                      |                  |
|   | 20    | ZAP-70    | x sc-1526     | x             | x           |                          |                      |                  |
|   | 21    | Fyn       | x sc-16       | x             | x           |                          |                      |                  |
|   | 22    | IRS-1     | x sc-559      | x             | x           |                          |                      |                  |
|   | 23    | Crk-L     | x sc-319      | x x x        | x x x       |                          |                      |                  |
|   | 24    | ERK-1     | x sc-94       | x             | x           |                          |                      |                  |
|   | 25    | Lck       | x sc-433      | x             | x           |                          |                      |                  |
|   | 26    | Raf-1     | x sc-133      | x x x        | x x x       |                          |                      |                  |
|   | 27    | NFκB p65  | x sc-8008     | x NS NS      | x NS NS     |                          |                      |                  |
|   | 28    | Cbl-b     | x sc-8006     | x             | x           |                          |                      |                  |
| Group C | 29    | cyclin A  | sc-596       | x             | x           |                          |                      |                  |
|   | 30    | MEK-2     | sc-13159     | NS NS        | NS NS       |                          |                      |                  |
|   | 31    | NFκB p50  | sc-8414      | NS NS        | NS NS       |                          |                      |                  |
|   | 32    | c-Jun     | sc-7481      | x             |             |                          |                      |                  |
|   | 33    | Pan-cytokeratin | sc-8018 | x             |             |                          |                      |                  |
|   | 34    | MEK-1     | sc-219       | x x x        | x x x       |                          |                      |                  |
|   | 35    | Ini-1     | sc-13055     | x             |             |                          |                      |                  |

a 1–16, identified in this study and shown in Fig. 4; 17–18, additionally identified by Salomon et al. (19); 19–28, reported to be Tyr-phosphorylated in some cells (see footnote d); 29–35, not known to be Tyr-phosphorylated.

b Matching peptides in MALDI (e.g., 14/29 indicates 14 matching peptides out of a total 29 used for the PMF search); sequence coverage (%).

c Salomon et al. (19).

d References 7, 9, 19, 48, 51, 52, and 59–66.

e Obtained from Santa Cruz Biotechnology.

f Protein capture on anti-p-Tyr mAb (PY-KD1) beads and identification by Western blotting (WB) using protein-specific Abs.

g,h IP, immunoprecipitation; WB, Western blotting; NS, Ab is not suitable for IP according to the manufacturer's specifications.

i Identified after subtraction of all peptides corresponding to primary component in the band.
cancer drug STI-571 (Gleevec) (8, 9), RT10+ cells provide a convenient model system to analyze down-regulation. On the other hand, activation of the EGFR, a tyrosine kinase, in serum-starved HeLa cells has often been used to analyze induction of a subset of the tyrosine phosphoproteome (14, 15). Both types of cells were therefore grown in culture and separate aliquots treated with either EGF or Gleevec for 1 or 2 h, respectively. Lysates of both treated and untreated cells were then analyzed by gel electrophoresis and immunoblotting with the PY-KD1 mAb. Optimization experiments suggested that about 10- to 100-fold more protein from the EGF-treated HeLa cells was required to get comparable signals as for RT10+ cells. As expected, all major bands were substantially reduced in protein extract from Gleevec-treated RT10+ cells and enhanced in EGF-treated HeLa cells as compared with the respective untreated controls (Fig. 5). Similar experiments were then carried out using our 35-feature Ab microarrays (Table II) and the anti-p-Tyr mAb for sandwich-type detection.

Equal amounts of RT10+ protein extracts, before and after Gleevec treatment, were captured on arrays and probed with Cy5-labeled PY-KD1 mAb. In keeping with the pilot experiment results, the optimum total protein concentration was again 10 ng/ml, with a 0.1-ml volume consumed per chip. No signals were observed upon binding of the mAb to the spotted array in the absence of cell extract, nor when the protein extract had been treated with protein tyrosine phosphatase prior to analysis (data not shown). Representative images of Ab features from two arrays, incubated with either “before” or “after” extracts, are shown pairwise in Fig. 6 for each cognate protein. Arbitrary signals from extracts of untreated cells were in the range of 2–14 for all proteins (Figs. 6 and 7), except for Ini-1 (<2.0), which we considered below the detection limit and was therefore not included in the figures. Arbitrary signals from extracts of Gleevec-treated cells ranged from 1 to 7 (Fig. 6). The ratio of arbitrary signals obtained from extracts of untreated and treated cells (“Rr” or “fold reduction by Gleevec” in Fig. 7) was found to be over 2.0 for 18 proteins, between 1.8 and 2.0 for five proteins, between 1.5 and 1.8 for two proteins, and below 1.5 for nine proteins. Twelve of the 16 proteins (75%) identified by MS in this study and represented on this particular array (Table II, group A, 1–16; red dots in Fig. 7) had an Rr > 2.0, and all but one had an Rr > 1.8. The highest Rr was observed for c-Abl (3.2) and the lowest Rr for SLP-76 (1.63). Twenty-two of the 28 proteins (79%) that had never been reported as Tyr-phosphorylated (Table II, group C, no. 29–24; green dots in Fig. 7) all had Rr < 1.5. Virtually all the red and purple dots in Fig. 7 are in the top half of the plot, indicating medium to high initial arbitrary signals that were substantially down-regulated as the result of Gleevec treatment. The green dots cluster at the bottom-left, indicating low to medium arbitrary signals that did not change much in response to the drug. In separate experiments performed with identical batches of protein extracts, arbitrary signals for the same proteins, from comparable cell states (i.e. pre- or post-Gleevec), varied less than 15% (data not shown).

Extracts from untreated and EGF-stimulated cells were also

**Fig. 5. Effects of Gleevec and EGF on the Tyr phosphoproteome in RT10+ and HeLa cells.** RT10+ cells were treated with 1 μM Gleevec for 2 h; serum-starved HeLa cells were treated with 150 ng/ml EGF for 1 h. Equal amounts of protein extract from treated and untreated cells (2 μg/lane for RT10+ cells; 20 μg/lane for HeLa cells) were separated by gel electrophoresis. **Upper panel,** Western blotting using the anti-p-Tyr PY-KD1 mAb. **Lower panel,** Coomassie-stained gel showing equal loading of “before and after” samples of each cell type, and a 10-fold larger load of HeLa extracts as compared with RT10+ extracts.
incubated with microchips containing the same 35 pre-spotted Abs (Table II), and detection of phosphorylation state was again achieved by subsequent probing with Cy5-labeled anti-p-Tyr PY-KD1 mAb (Fig. 8). The optimal total protein concentration in this experiment was 1 μg/ml, about 100-fold higher than for RT10 cells. Arbitrary signals from untreated extract were extremely low, generally below 2.0 with only a few exceptions (Fig. 8). Arbitrary signals from EGF-treated extract ranged from 2.0 to 4.5 for 29 out of 35 proteins (83%) (Fig. 9). Six proteins (Cas-L, Crk-L, Dock 180, IRS-1, PLC-γ1, Ras-GAP) with arbitrary signals of less than 2.0 were considered to be below detection limit, even after EGF stimulation. Ratios of arbitrary signals from extracts of treated and untreated cells (\(R_i\) or “fold induction by EGF” in Fig. 9) were as follows: over 2.0 for 15 proteins, between 1.8 and 2.0 for two proteins, between 1.5 and 1.8 for five proteins, and below 1.5 for seven proteins. The highest \(R_i\) was observed for Cbl (3.3). Twelve of the 22 proteins (55%) that had been identified as Tyr-phosphorylated in various cell types displayed \(R_i > 2.0\). One group of proteins without any known Tyr phosphorylation

Fig. 6. Inhibition of the Bcr-Abl-kinase in RT10+ cells by Gleevec represses the Tyr phosphorylation state of selected proteins. RT10+ cells were treated with 1 μM Gleevec for 2 h, and protein extracts (10 ng/ml total protein) were captured on arrays containing 35 Abs against specific proteins and probed with Cy5-labeled anti-p-Tyr PY-KD1 mAb (5 μg/ml). Images of representative Ab features from individual arrays, incubated with extracts from untreated or treated cells, are shown for each cognate protein. Numbers represent mean arbitrary signal (±S.D.), calculated for six to nine replicates of Ab elements on individual arrays.
sites (Cytokeratins, Ini-1, and NFκB p50) had $R_0 < 1.5$, as expected. Cyclin A, MEK-1, MEK-2, and c-Jun, on the other hand, represented a different group of proteins not known to be Tyr-phosphorylated but with $R_0 > 1.8$. The synopsis is that, in contrast to the RT10+ cells/Gleevec system, the red and green dots are all over the place in the plot of Fig. 9, indicating a substantially different targeting of the EGFR and Bcr-Abl protein tyrosine kinase activities in vivo, as has already been well documented in the scientific literature by other means (14, 19).

Confirmation of Quantitative Changes in Tyr Phosphorylation by Immunoblotting—To further confirm some of the above Ab microarray results, the Tyr phosphorylation state of two selected proteins, one in each cell type, was experimentally verified by comparative immunoblotting. To this end, PLC-γ1 and SHIP-1 were immunoprecipitated from, respectively, RT10+ and HeLa cells, each before and after treatment with, respectively, Gleevec and EGF. Precipitates were analyzed by gel electrophoresis and Western blotting, first with “self” Ab and then, on equal aliquots, with PY-KD1 mAb. The results presented in Fig. 10 show that the protein levels did not change in either cell type after 1–2 h of treatment and that equal amounts had been loaded in each lane. Anti-p-Tyr blots, on the other hand, clearly indicated changing levels of this modification. p-Tyr levels of PLC-γ1 and SHIP-1 were, respectively, down 2.5-fold and up 1.8-fold, which compares reasonably well with the down- and up-regulation of the arbitrary signals recorded in the p-Tyr array analyses by, respectively, 2.8 and 2.7.

DISCUSSION

In order to examine changes in protein phosphorylation at Tyr by sandwich-based Ab microarray analyses, we have generated a mAb, PY-KD1, that recognizes p-Tyr in a sequence-independent manner and without cross-reactivity, even at high concentrations. We report here a multi-step hybridoma screening procedure by ELISAs utilizing a combination of 20 peptide haptens. This strategy was successfully applied to eliminate negative hybridomas and clones secreting low-affinity, cross-reactive, or sequence-dependent Abs.

Differences in reactivity of the PY-KD1 mAb and its commonly used commercial counterparts (PY-100, 4G10, and PY-20) were observed in ELISAs, and indicated that PY-KD1 is one of the few if not the only available anti-pTyr mAb right now with negligible affinities for p-Ser, p-Thr, and nonphosphorylated Tyr. In assays typically requiring high Ab titers, as for example in immunohistochemistry or protein arrays, such cross-reactivity of the commercial anti-p-Tyr mAbs may become problematic, all the more so considering that Ser and Thr phosphorylation sites and nonphosphorylated Tyr residues in proteins are far more abundant than p-Tyr. This situation may also apply to affinity immunocapture procedures whereby the highest possible quantity of Ab has been immobilized to yield high-ligand density, high-capacity particles, as was the case in a component of this study.

Pilot Ab array experiments suggested the presence of several cognate proteins in RT10+ cells with a different degree of Tyr phosphorylation. Interestingly, the micro-sandwich assay with Cy5-PY-KD1 (for p-Tyr) was 1,000 times more sensitive than the assay with direct Cy5-labeling (for protein) of the extract. Similar limitations in sensitivity of direct fluorescent labeling of proteins have previously been reported (27, 30, 40, 50). To ensure adequate sensitivity of Tyr phosphorylation microarray analyses, we used an excess of detection Ab. At 5 μg/ml Cy5-PY-KD1, detection could be readily achieved using only 10 ng/ml total protein from RT10+ cells. Consistent with Western blotting results (Fig. 5), a substantially higher total

![Fig. 7. Comparative ratiometric plot of Ab microarray arbitrary signals reflecting suppression of Tyr phosphorylation in RT10+ cells by Gleevec. RT10+ cells were treated with 1 μM Gleevec for 2 h, and detection of protein Tyr-phosphorylation state was performed utilizing the sandwich Ab microarray platform. Mean arbitrary signals were determined for each Ab element on the microarrays that had been incubated with extracts from untreated and treated cells. Fold reduction by Gleevec, $R_0$, is the ratio of arbitrary signals obtained for untreated ($S_u$) and treated ($S_t$) extracts, $R_0 = S_u/S_t$. Each colored dot represents features corresponding to a single Ab on the microarrays. Numbering of the Abs and the names of the cognate proteins are given in Table II. Color coding is as follows. Group A: red, proteins in Bcr-Abl expressing cells, purified using the anti-p-Tyr Abs and identified by MS in this study (see Fig. 4 and Table II); magenta, similar proteins additionally identified by Salomon et al. (6). Group B: yellow, other proteins known to be Tyr phosphorylated. Group C: green, proteins that have not been reported to be Tyr-phosphorylated. Experimental details are as listed under Fig. 6.](image-url)
protein concentration, 1 μg/ml, was required to generate detectable signals on a p-Tyr chip when using extracts from EGF-treated HeLa cells. Of course, the sensitivity of the p-Tyr microarray platform is entirely dependent on the overall abundance of Tyr-phosphorylated proteins in the sample and therefore largely cell-type specific. Even so, the sensitivities we have observed for p-Tyr detection with our system so far are remarkable by any measure of post-translational modification analysis, and even for protein analysis in general.

Using array incubation volumes of ~100 μl, total protein amounts consumed per chip range from 1 to 100 ng depending on cell type. In the case of RT10+ and HeLa cells, lysates of 1 × 10^7 cells yield typically 1–2 mg of total protein, in a 1-ml volume, that is then serially diluted 1,000-fold (HeLa extract) or 100,000-fold (RT10+ extract) to a final concentration of 1 μg/ml or 10 ng/ml, respectively. This is the equivalent of a 1-ml extract made from either 10,000 (HeLa) or as little as 100 RT10+ cells; each sufficient for 10 microarray analyses. Conceivably, an even smaller number of cells could be lysed in sub-milliliter volumes to yield identical protein concentrations, but these experiments have yet to be done. It should be noted that low-volume processing of very small numbers of cells could pose specific experimental problems not encountered during larger-scale sample preparation followed by serial dilution. But even when future developments would not match the most optimistic projections, the required number of cells would still be in marked contrast to those in other types of tyrosine phosphoproteome profiling, such as affinity capture.

**FIG. 8.** EGF treatment of serum-starved HeLa cells elevates the Tyr phosphorylation state of selected proteins. Subconfluent HeLa cells were grown in serum-free medium for 24 h and stimulated with 150 ng/ml EGF for 1 h. Protein extracts (1 μg/ml total protein) were captured on arrays containing 35 Abs against specific proteins and probed with Cy5-labeled, anti-p-Tyr PY-KD1 mAb (5 μg/ml). Images of representative Ab features from individual arrays, incubated with extracts from untreated or treated cells, are shown for each cognate protein. Numbers represent mean arbitrary signal (±S.D.), calculated for six to nine replicates of Ab elements on individual arrays.
in combination with mass spectrometric identification which, in fact, was also used as part of this study. In those studies, both in our laboratory and elsewhere, typically $10^7$ to $5 \times 10^8$ cells have been used (14, 15, 21). Compared with MS-based methods, the Ab array method falls significantly short at this time in terms of protein coverage and mapping of the p-Tyr sites; i.e. only dozens of cognate proteins are being profiled instead of the entire cell population, without any information on possible modification sites. With this in mind, we have tried to at least maximize our chances for getting broader p-Tyr protein coverage by carefully selecting the Abs for printing. Besides sensitivity, the benefits of our Ab array approach are a capacity for i) improved sample throughput, ii) virtually unlimited antigen multiplexing by increasing the number of Ab features deposited on the chip, and iii) multiplexing of the sandwich-based detection by using two or more differentially labeled affinity probes.

Whereas a quantitative Tyr phosphorylation microarray platform would be a welcome addition to the bio-analytical toolbox, such process requires calibration against standards, for example recombinant or purified p-Tyr proteins, as in a conventional ELISA. Unfortunately, those specific standards are not readily available. Therefore, at this time, the p-Tyr microarray platform described herein is only applicable to a comparative analysis of two or more samples rather than exact quantitation of phosphorylation of individual proteins. We also cannot account for the possible presence of multiple, often independently changing p-Tyr sites on individual proteins. In addition, the arbitrary signal for any particular protein is strongly dependent on the properties of the capturing Ab, as well as on variously induced changes (e.g. by phosphorylation itself) in three-dimensional structure that could affect binding to the Ab, making it difficult to compare phosphorylation states of the different cognate proteins captured by the various Abs on the same chip. Instead, this platform is de-
Tyrosine Phosphoproteome Analysis

signaled to specifically cross-compare each individual member of a set of proteins between two or more samples, whereby corresponding proteins are captured from the different lysates on separate chips but using the exact same Abs under the same conditions.

Evaluation of a 35-feature, p-Tyr microarray platform demonstrated its utility in monitoring both up- and down-regulation of the Tyr phosphoproteome. A ratiometric approach was used to describe changes in Tyr phosphorylation of two protein populations by calculating ratios of arbitrary signals from control and treated samples or vice versa ($R_{ij}$ for negative regulation or $R_{ij}$ for positive regulation). Microarray analyses of RT10+ cells treated with Gleevec and HeLa cells treated with EGF indicated that $R_{ij}$ or $R_{ij}$ values of 1.8 or more suggest a good probability of changes in Tyr phosphorylation. By contrast, $R_{ij}$ or $R_{ij}$ values of 1.5 or less denote the likely absence of such differences. Some proteins had $R_{ij}$ or $R_{ij}$ values between 1.5 and 1.8, making it difficult to deduce if their p-Tyr levels had changed, quite likely due to limitations in sensitivity. It is important to understand that, depending on the experimental system and objectives, each study will require its own threshold ($R_{ij}$ or $R_{ij}$ value) to qualify as a valid indicator of changes in protein phosphorylation. In certain situations it may be reasonable to apply a more stringent approach by choosing $R_{ij}$ or $R_{ij}$ values of 2.0 or more as a “difference threshold.”

Tyr phosphorylation array data generated for Bcr-Abl-expressing cells treated with a specific kinase inhibitor was in good agreement with the extensive body of knowledge that exists for this experimental system. Most proteins that had reduced levels ($R_{ij} > 1.8$) of phosphorylation after exposure to Gleevec had previously been reported as direct substrates of the Bcr-Abl tyrosine kinase, including PI3K, SHIP-1, SHIP-2, PLCγ1, Dok-1, Dok-2, Cbl, Crk-L, Shc, RasGap, and Irs-1 (7, 9, 51), or to be down-regulated by Gleevec in cells transfected with a p210Bcr/Abl-expressing plasmid, including c-Abl, SHIP-2, SH-PTP2, Gab-1, Dok-1, Irs-1, and Erk-1 (19, 51, 52).

In conventional affinity methods for p-Tyr protein enrichment, such as immunocapture or precipitation, it is generally accepted that not all detected proteins are phosphorylated, as unmodified proteins can bind to and co-precipitate with the labeled p-Tyr-specific detection mAb and describe a general-ized, ratiometric approach to analyze data generated by this assay. Our approach offers an advantage of extremely low

In conclusion, we have designed and successfully tested a sandwich Ab microarray platform utilizing a fluorescently labeled p-Tyr-specific detection mAb and describe a general-ized, ratiometric approach to analyze data generated by this assay. Our approach offers an advantage of extremely low
sample and reagent consumption, scalability, detection multiplexing, and potential compatibility with micro-fluidic devices and automation. Future work will focus on development of potential applications of this platform to define signal transduction pathways, for molecular classification of tumors, compound profiling and Toxicology studies, and analysis of patients’ individual sensitivities to tyrosine kinase inhibitors, such as the efficacy of Gleevec (8) or Gefitinib (11) targeted cancer therapy, among many.

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Molecular & Cellular Proteomics 3.11
Tyrosine Phosphoproteome Analysis

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