Multiplex Analysis of Intracellular Signaling Pathways in Lymphoid Cells by Microbead Suspension Arrays*

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Phosphorylation analysis of signaling proteins is key for examining intracellular signaling pathways. Conventional biochemical approaches, e.g. immunoprecipitation, Western blot, and ELISA, have played a major role in elucidation of individual signaling events. However, these methods are laborious, time-consuming, and difficult to adapt for high throughput analysis. A multiplex approach to measure phosphorylation state of multiple signaling proteins simultaneously would significantly enhance the efficiency and scope of signaling pathway analysis for mechanistic studies and clinical application. This report describes a novel multiplex microbead suspension array approach to examine phosphoproteomic profiles in lymphoid cells. In the Jurkat T-cell leukemia line, the multiplex assay enabled targeted investigation of phosphorylation kinetics of signal transduction from receptor proximal events (tyrosine phosphoproteins CD3, Lck, Zap-70, and linker for T-cell activation) to cytosolic events (serine/threonine phosphoproteins Erk and Akt) to transcription factors (serine/threonine phosphorylated Rsk, cyclic AMP-response element-binding protein, and STAT3). To broaden the application of the multiplex analysis, signaling pathways were also studied in B-cell lymphoid tumor lines that included chronic lymphocytic leukemia lines. In these cell lines, multiplex suspension array enabled phosphoproteomic analysis of signaling cascade mediated by Syk, a homolog of Zap-70. Results obtained by multiplex analysis were confirmed by immunoprecipitation and Western blot methods. The examples of T-cell and B-cell signaling pathway analyses in this report demonstrate the utility of the multiplex suspension arrays to investigate phosphorylation dynamics and kinetics of several signaling proteins simultaneously in signal transduction pathways. Molecular & Cellular Proteomics 5:758–768, 2006.

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Regulated phosphorylation of signaling proteins in cells plays a critical role in signal transduction from the surface receptor along a pathway that produces a physiological response in the cell, such as a novel pattern of gene expression with subsequent effects on cell function (1). Abnormally high activation, or lack of activation of certain signaling proteins, may interfere with proper functioning of the signal transduction apparatus in a cell. Such anomalies may result in disease (2, 3).

In cells of the immune system, tight regulation of cellular signaling events orchestrates a finely tuned signaling system that leads to specific and measured responses to pathological challenges (4). Additionally cell signaling plays a key role in the maturation of lymphoid cell populations during developmental progression (4–7). In the T-cell signaling pathway, a signal emanates from the T-cell receptor (TCR)† on the cell surface and is transduced across the cell membrane to the cytosol via a series of signaling proteins. TCR-proximal events are carried out by regulated phosphorylation of several proteins, Lck, Zap-70, and LAT (8, 9). A key target for activated Zap-70 kinase is LAT, a palmitoylated membrane-associated adapter protein (10, 11), which functions as a platform for the assembly of other key signaling proteins, including SLP-76, Vav, and phospholipase C-γ (10, 12–14). This multiprotein complex serves as a hub, coupling the signal received from the TCR to cytosolic components of the T-cell activation pathway. Downstream of the LAT complex, the signal leads to rearrangement of the actin cytoskeleton via phosphorylation of Wiscott-Aldrich syndrome protein and to transcriptional activation via the Erk pathway (15). Subsequent activation of specific transcription factors (including Rsk, CREB, and STAT3) results in expression of several cytokines including interleukin-2, a hallmark of T-cell activation (16, 17). In B-cells, signaling initiates

1 The abbreviations used are: TCR, T-cell receptor; BCR, B-cell receptor; B-CLL, B-cell chronic lymphocytic leukemia; CREB, cyclic AMP-response element-binding protein; Erk, extracellular signal-regulated kinase; IgG, immunoglobulin G; IgM, immunoglobulin M; IP, immunoprecipitation; LAT, linker for T-cell activation; Lck, lymphocyte-specific kinase; MFI, median fluorescence intensity; STAT3, signal transducers and activators of transcription 3; Syk, Syk/Zap-70 family of kinases; Zap-70, ζ-associated protein; WB, Western blot; MAP, mitogen-activated protein; MAPKAP, MAP kinase-activated protein.
through the B-cell receptor (BCR) complex on the cell surface (18, 19), and the activation cascade utilizes the Syk tyrosine kinase, a homolog of Zap-70 tyrosine kinase. Subsequent signaling events, including phosphorylation of Akt, Erk, and CREB, are shared not only between B- and T-cells but also other cell types (20).

For elucidating basic molecular mechanisms underlying cellular signaling, conventional biochemical and cell biological methodologies, such as immunoprecipitation, Western blot, and immunofluorescence, have been instrumental. However, investigation of complex signaling pathways, such as those regulating lymphoid cell activation, requires the development of more efficient approaches to better define the relationships of signal transduction pathways and multiple outcomes (21, 22). Novel approaches that will enable comprehensive measurement of signaling kinetics, magnitude of signal, and pathway cross-talk will also lead to a better understanding of cellular signaling in both normal cells and cells displaying abnormal functions, including tumor cells.

This report describes a multiplex microbead suspension array approach for analysis of phosphorylation of multiple signaling proteins by simultaneous detection in a single sample. We evaluated the multiplex microbead suspension array technology developed by Luminex Corp. (Austin, TX) termed multianalyte profiling (Lum-MAP). This technology utilizes mixtures of unique fluorochrome-coded sets of polystyrene microbead suspension (5.6 μm in diameter) to detect specific target analytes (23, 24). Individual microbead sets are coated with specific capture molecules, e.g. antibodies. Multiplex capability involves mixing several populations of these fluorescent coded microbeads, each coated with a specific capture antibody, into one reaction vessel at the start of the test. Subsequently sample (e.g. tumor cell lysate containing target proteins) is incubated with this microbead mixture. Analytes are captured by the relevant antibodies on the microbeads. Detection of the analytes is performed by the addition of secondary reagent(s) attached to reporter fluorochrome (e.g. phycoerythrin). Finally analysis is performed in the Luminex flow cytometer where lasers and digital signal processing methods identify the bead set and detect the reporter. This approach is rapid and sensitive and lends itself to high throughput in an economical fashion.

The key element of our novel multiplex microbead suspension array immunoassay is the detection of tyrosine phosphorylation of multiple proteins by the use of a single anti-phosphotyrosine antibody. In addition, for the detection of selected serine/threonine phosphorylated signaling proteins, anti-phosphoprotein-specific antibodies were used. We studied T-cell activation pathway in the Jurkat T-cell leukemia line as a model system (25, 26). This novel multiplex microbead suspension array system enabled analysis of the dynamics and kinetics of Jurkat T-cell signaling by the detection of phosphorylation of protein kinases (Lck, Zap-70, Syk, Akt, and Erk), an adaptor (LAT), and transcription factors (Rsk, CREB, and STAT3) in a single cell lysate sample. In addition, this multiplex suspension array immunoassay was applied to the analysis of signal transduction in B-cells, including the Ramos line derived from an Epstein-Barr virus lymphoma and several cell lines from patients with B-cell chronic lymphocytic leukemia (B-CLL). Expression of Zap-70 in leukemic cells of B-CLL patients is of prognostic value for aggressive disease and shorter patient life span (27, 28). Importantly our findings with the multiplex microbead suspension arrays for these phosphoproteins were confirmed by conventional methods of immunoprecipitation and Western blotting.

EXPERIMENTAL PROCEDURES

Cells—The human T-cell leukemia Jurkat (E6-1) cell line and Burkitt lymphoma Ramos (RA 1) B-cell line were obtained from ATCC (Manassas, VA). CLL cell lines Mec-1 and Mec-2 (29) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GbH (56). The CLL cell line MO1043 (30) was a gift from Dr. Ricardo Dela-Favera (Columbia University, New York, NY). These CLL lines are of B-cell origin derived from human chronic B-cell leukemia patient blood. By flow cytometry, using fluorescent labeled antibodies, we verified the above cell lines to be positive for B-cell markers CD19 and CD20. Jurkat, Ramos, and MO1043 cells were propagated in RPMI 1640 medium containing 10% fetal bovine serum. Mec-1 and Mec-2 cells were propagated in Iscove’s modified Dulbecco’s medium containing 10% fetal bovine serum.

Antibodies and Reagents—Monoclonal antibodies against signaling proteins Lck, Zap-70, LAT, and Syk and biotinylated anti-phosphotyrosine monoclonal antibody (4G10) were supplied as purified IgG by Upstate USA (Charlottesville, VA). The following BeadmatesTM (capture beads and biotinylated reporter antibody pairs) were also obtained from Upstate USA: phospho-Akt/PKBα (Ser-473), phospho-Erk/MAP kinase 1/2 (Thr-185/Tyr-187), phospho-Rsk MAPKAP kinase 1α (Ser-380), phospho-STAT3 (Ser-727), and phospho-CREB (Ser-133). In addition, capture and reporter antibodies from the above Beadmates (Upstate USA) were used for immunoprecipitation and Western blot analysis. Antibodies for the detection of total proteins (Lck, Zap-70, Syk, LAT, Erk, Akt, Rsk, CREB, and STAT3) in Western blots were also from Upstate USA. Anti-CDS2 antibody UCHT1 and its isotype control antibody were obtained from BD Pharmingen. Streptavidin-conjugated R-phycocerythin was from CalTag (Burlingham, CA). Anti-IgM antibody was purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Protease inhibitor mixture and purified 10% Nonidet P-40 were purchased from Roche Applied Science. Protein G-conjugated Sepharose was from Sigma.

Treatment of Lymphoid Cells—Jurkat (E6-1) T-cells, Ramos B-cells, and the CLL cell lines (Mec-1, Mec-2, and MO1043) were treated with sodium pervanadate to inhibit intracellular tyrosine phosphatases, resulting in hyperphosphorylation of tyrosine kinases and their substrates including serine/threonine kinases. Sodium pervanadate was prepared by adding 330 μl of 30% hydrogen peroxide to 1 ml of 20 mM sodium vanadate (pH 10.0). Cells were resuspended to a density of 1 × 10⁶ cells/ml in plain RPMI 1640 medium and prewarmed to 37 °C for 5 min. To each milliliter of cell suspension, 40 μl of sodium pervanadate was added. Cells were mixed and immediately incubated at 37 °C for 5 min. Treated cells were lysed, and cell lysates were processed as described below.

To examine intracellular signaling kinetics using anti-CDS2 antibody, Jurkat cells were resuspended in RPMI 1640 medium at a density of 1 × 10⁶/ml and prewarmed to 37 °C for 5 min. Anti-CDS2 antibody (UCHT1) was added to a final concentration of 5 μg/ml. Cells were
mixed and incubated at 37 °C for various times ranging from 15 s to 1 h. Treatment was terminated by the addition of cell lysis buffer as described below.

Preparation of Cell Lysates—Cells were lysed by adding lysis buffer (2% Nonidet P-40, protease inhibitor mixture (both from Roche Applied Science, in PBS), and 0.5 mM sodium orthovanadate). Lysate was immediately vortexed and incubated on ice for 15 min. Cell debris were removed by low speed centrifugation. Total protein concentration of lysates was determined by BCA reagent kit (Bio-Rad). Lysates were stored frozen at −80 °C until used.

Coating Microbeads with Antibodies—Monoclonal antibodies against CD3, Lck, Zap-70, LAT, and Syk were coated as capture antibodies on individual sets of microbeads. In addition, one bead set was coated with BSA as a control for nonspecific interactions. Another bead set was coated with biotin-conjugated goat IgG (Jackson Immunoresearch Laboratories, Inc.), a positive control for the reporter molecule (streptavidin-conjugated phycoerythrin). Luminex beads were coated with the above proteins as described previously (31). Briefly bead stock was resuspended by vortexing and sonication (15–30 s). An aliquot of 2.5 × 10^6 beads was removed and centrifuged at 12,000 × g for 2 min. Beads were resuspended in 80 μl of activation buffer (100 mM monobasic sodium phosphate, pH 6.3) by vortexing and sonication (15–30 s). To activate the beads for cross-linking to proteins, 10 μl of 50 mg/ml N-hydroxysulfosuccinimide (Pierce) was added, and beads were mixed by vortexing. Then 10 μl of 50 mg/ml 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (Pierce) was added, and beads were mixed again by vortexing. The bead mixture was shaken on a rotator at room temperature for 20 min and centrifuged at 12,000 × g for 3 min. Beads were washed twice with 1 ml of 50 mM MES (pH 6.0) buffer. To coat with antibody, pelleted beads were resuspended in the relevant antibody solution (25–100 μg/ml) in 50 mM MES (pH 6.0) buffer. The mixture of activated beads and antibodies was incubated by shaking on a rocker for 2 h at room temperature for coupling. After coating, beads were washed twice with wash buffer (0.1% Tween 20 in PBS, pH 7.40) and resuspended in 1 ml of blocking buffer (1% BSA, 0.1% Tween 20 in PBS, pH 7.4, 0.05% sodium azide). Blocking was performed by shaking on a rocker at room temperature for 30 min. After blocking, beads were washed twice in 1 ml of blocking buffer. Finally antibody-coated beads were resuspended in 1 ml of blocking buffer and stored at 4 °C for up to a week. For long term storage, beads were kept frozen at −70 °C for several months.

Microbead Suspension Array Immunooassay of Signaling Proteins— Immunoreactions were set up in 96-well, filter-bottomed plates (Nunc). To each well, 25 μl of cell lysate (0.4 mg/ml total protein) was added, and beads were mixed again by vortexing. The bead mixture was shaken on a rotator at room temperature for 20 min and centrifuged at 12,000 × g for 3 min. Beads were washed twice with 1 ml of 50 mM MES (pH 6.0) buffer. To coat with antibody, pelleted beads were resuspended in the relevant antibody solution (25–100 μg/ml) in 50 mM MES (pH 6.0) buffer. The mixture of activated beads and antibodies was incubated by shaking on a rocker for 2 h at room temperature for coupling. After coating, beads were washed twice with wash buffer (0.1% Tween 20 in PBS, pH 7.40) and resuspended in 1 ml of blocking buffer (1% BSA, 0.1% Tween 20 in PBS, pH 7.4, 0.05% sodium azide). Blocking was performed by shaking on a rocker at room temperature for 30 min. After blocking, beads were washed twice in 1 ml of blocking buffer. Finally antibody-coated beads were resuspended in 1 ml of blocking buffer and stored at 4 °C for up to a week. For long term storage, beads were kept frozen at −70 °C for several months.

Microbead Suspension Array Immunooassay of Signaling Proteins— Immunoreactions were set up in 96-well, filter-bottomed plates designed for high throughput separations (1.2-μM MultiScreen, Millipore Corp.). Microbeads (2000 beads of each set) coated with a specific antibody were mixed. This multiplex, microbead mixture was added to each well. To this, 25 μl of cell lysate (0.4 mg/ml total protein; corresponding to approximately 8 × 10^6 cells) was added. Performance of antibody-coated microbeads on serial dilutions of cell lysates was tested. The lysate concentration used in this study is within the linear range of signal responses of the microbead sets (data not shown). The contents were mixed at 1400 rpm on a plate shaker (Labnet International Inc., Woodbridge, NJ) for 2 h at room temperature. After incubation with the lysate, liquid was drained from the bottom of the plate under vacuum. The microbeads were washed twice by adding 150 μl of wash buffer/well and draining out under vacuum successively. For detection of tyrosine phosphorylated signaling proteins bound to antibodies coated on microbeads, 25 μl of biotinylated anti-phosphotyrosine antibody 4G10 (0.5 μg/ml in wash buffer) was added as the detection reagent. Antibody 4G10 displayed excellent sensitivity at this concentration for the detection of phosphotyrosine residues with no cross-reactivity to phosphoserines/phosphothreonines (21). Microbeads were mixed as before and incubated at room temperature for 30 min. Microbeads were washed twice as before. To detect biotinylated 4G10, streptavidin-conjugated R-phycocerythrin was added at a dilution of 1:1000 in wash buffer as the reporter molecule and incubated for 15 min at room temperature. Microbeads were washed once with wash buffer, resuspended in 100 μl of wash buffer/well, and analyzed in the Luminex-100™ instrument. Beadmate kits were used according to the manufacturer’s instructions (Luminex Corp.).

Luminex-100 Operation and Data Analysis—The Luminex-100 instrument was used at default settings, set by the manufacturer for routine applications, as directed by the user’s manual. Data were acquired by Luminex Data Collection Software (Version 1.7). This software package was used according to instructions in the user’s manual supplied by the manufacturer for routine operation of the instrument, data acquisition, and data analysis. The instrument was calibrated with Calibration Beads supplied by the manufacturer to adjust the settings for bead set identification or “Classification” and for the detection of “Reporter” (phycocerythrin). Events were gated to exclude doublets and other aggregates. A hundred independent, gated events were acquired for each bead set. The median fluorescence intensity (MFI) or “signal” of a hundred events (beads) was used as a measure of the detection of protein phosphorylation. After acquisition by Luminex software, the data were further processed by Microsoft Excel software.

Immunoprecipitation and Western Blotting—Immunoprecipitation was performed by mixing capture antibody (5 μg/ml of purified IgG) with 0.5 ml of cell lysates (1 mg/ml total protein) on a rotator for 1 h at 4 °C. Protein G-conjugated Sepharose was added (25 μl of pre-swollen gel/ml) and mixed on rotator for 30 min at 4 °C. Sepharose beads were washed three times in wash buffer (PBS containing 1% Tween 20) and resuspended in 40 μl of SDS sample buffer (Novex 2× SDS, Tris-glycine sample buffer with 0.71 mM β-mercaptoethanol; Invitrogen). The samples were boiled for 5 min. The immunocomplexes were resolved on SDS-polyacrylamide gels (Novex 8–16% precast gradient Tris-glycine gels). Resolved proteins were transferred to PVDF membranes. To detect tyrosine phosphorylation, biotin-conjugated anti-phosphotyrosine antibody (4G10, 0.5 μg/ml) was used. For the detection of serine/threonine phosphorylation, biotinylated antibodies from the respective Beadmates (phospho-Akt/PKBα (Ser-473), phospho-Erk/MAP kinase 1/2 (Thr-185/Tyr-187), phospho-Rsk MAPKAP kinase 1a (Ser-380), phospho-STAT3 (Ser-727), and phospho-CREB (Ser-133) were used according to the manufacturer’s instructions (Upstate USA). Blots were developed with Vectastain ABC detection reagent (Vector Laboratories, Burlingame, CA) and ECL Plus Western blotting detection system (Amersham Biosciences) and visualized on a Typhoon 9410 variable mode imager (Amersham Biosciences).

RESULTS

Phosphorylation of T-cell Signaling Proteins in Jurkat Cells—The multiplex microbead suspension array immunooassay was performed to detect phosphorylation of different cell signaling proteins in Jurkat T-cells treated with sodium per- vanadate. Treatment with pervanadate inhibits intracellular tyrosine phosphatases resulting in sustained phosphorylation of various tyrosine kinases and substrates reflecting the activation state of the cells (32). Tyrosine kinases so activated subsequently phosphorylate their substrates including certain downstream serine/threonine kinases resulting in their activa-

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2 I. H. Khan, S. Mendoza, P. Rhyne, M. Ziman, J. Tuscano, D. Eisinger, H.-J. Kung, and P. A. Luciw, unpublished.
tion (33). Ten sets of microbeads coated with monoclonal antibodies to signaling proteins (i.e., CD3, Lck, Zap-70, Syk, LAT, Erk, Akt, CREB, STAT3, and Rsk) were mixed and incubated with cell lysate. A microbead set coated with anti-Syk antibody was included as a specificity control because Syk protein is not expressed in Jurkat (E6-1) T-cells (34). Tyrosine phosphorylation of Lck, Zap-70, LAT, CD3, and Syk was detected by the anti-phosphotyrosine antibody (4G10), whereas serine/threonine phosphorylation of Erk, Akt, Rsk, CREB, and STAT3 was detected by anti-phosphoreporter antibodies (Beadmates). Proteins captured by microbeads coated with antibodies to Lck, Zap-70, LAT, and CD3 showed strong reaction to 4G10 antibody in lysate from pervanadate-treated Jurkat cells (Fig. 1A). Similarly proteins captured by microbeads coated with antibodies to Erk, Akt, Rsk, CREB, and STAT3 displayed a reaction to the relevant anti-phosphopeptide antibodies in lysates of the treated cells. Interassay variability was less than 10% (Fig. 1A, bars represent S.D.). This analysis showed that phosphorylated signaling proteins were readily detected by the multiplex microbead suspension array upon treatment of cells with sodium pervanadate. Interestingly constitutive phosphorylation of all the signaling proteins (i.e. in untreated cells) except Akt was relatively minimal (Fig. 1A). Constitutive phosphorylation of Akt in Jurkat T-cells has been reported previously (35). As expected, Syk phosphorylation was undetectable in Jurkat (E6-1) T-cells treated with sodium pervanadate (Fig. 1A). Data obtained by immunoprecipitation (IP) and Western blot (WB) confirmed that Syk was not detectable in these cells (Fig. 1B) as shown previously (34).

Antibodies used in coating fluorescent microbeads for capturing different signaling proteins in cell lysates were also tested by IP. Detection of the phosphorylated forms as well as the total amount of these proteins in lysates of Jurkat T-cells was performed by WB analysis of the IP complexes. Results of IP/WB analysis are presented in Fig. 1B. Together the multiplex and IP/WB analyses of Jurkat cells treated with sodium pervanadate demonstrated the following features. (a) Phosphorylated bands of the relevant proteins were detected only in lysates from cells treated with sodium pervanadate with the exception of Akt, which was phosphorylated in untreated cells as well. (b) Phosphorylated species of signaling proteins predominated in their respective immunoprecipitates. (c) The total amount of each signaling protein in the lysates from non-treated and treated cells was similar. The data presented here demonstrate the utility of multiplex microbead suspension array approach for analyzing phosphorylation of multiple signaling proteins simultaneously in a single sample.

Cell Signaling Kinetics in Jurkat T-cells Treated with Anti-CD3 Antibody—To study the kinetics of signaling pathway, Jurkat T-cells were treated with anti-CD3 antibody (UCHT1) for various times between 15 s and 1 h. Receptor-proximal signaling events were monitored by detecting tyrosine phosphorylation of Lck, Zap-70, Syk, and LAT. Results of this study are shown in Fig. 2. The multiplex microbead immunoprecipitation W374 Fig. 1. A, phosphoproteomic analysis by multiplex microbead suspension array immunoassay of signaling proteins in Jurkat T-cells. Cells were treated with sodium pervanadate, and cell lysates were prepared as described under “Experimental Procedures.” A mixture of microbeads coated with antibodies to CD3, Lck, Zap-70, Syk, LAT, Erk, Akt, CREB, STAT3, and Rsk were incubated with the cell lysates. Detection of phosphorylation was performed using a mixture of 4G10 antibody and protein-specific anti-phospho antibodies to Erk, Akt, CREB, STAT3, and Rsk. The results are an average of two independent experiments performed on duplicate samples in each experiment. S.D. is represented by error bars. B, immunoprecipitation and Western blot analysis of phosphorylation and total amount of signaling proteins in Jurkat cells. The sequence of proteins is the same as shown for multiplex suspension array immunoassay (A). Antibodies used for immunoprecipitation were the same as those coated on the microbeads for multiplex analysis. Western blot analyses were performed for the detection of protein phosphorylation (4G10 antibody and protein-specific anti-phospho antibodies) and total proteins as indicated.
and subsequently decayed to the preactivation levels in 30 min. The kinetics of phosphorylation of Lck and Zap-70 presented here correlated with those reported previously in a fluorescent imaging study of the immunological synapse using confocal microscopy (36). Syk is not expressed in Jurkat (E6-1) T-cells (34). Accordingly no increase in Syk phosphorylation, above the basal level observed in untreated cells, was detected during the 1-h period after the treatment with anti-CD3 antibody (Fig. 2). Syk phosphorylation thus served as an excellent internal control for the phosphoproteomic analysis of T-cell signaling pathway kinetics.

Signal transduction beyond these receptor-proximal events was also examined by the multiplex suspension array immunoassay, and the data are also shown in Fig. 2. Phosphorylation of Erk and Rsk was detected at 1 and 2 min postactivation, respectively. The signal for both of these serine/threonine kinases also decayed to the prestimulation levels in 1 h. An increase in phosphorylation of Akt over the basal level was detected after 2 min. However, in contrast to the other signaling proteins, phosphorylation levels of Akt continued to remain high for the duration of the period studied here (1 h). The above described multiplex suspension array immunoassay was also performed on lysates obtained from cells treated with an isotype control antibody for UCHT1 under the same time course conditions. In this control experiment, background reactivity of different microbead sets was similar to those with lysate from untreated cells (data not shown). In addition, the control BSA-coated beads yielded low background reactivity (MFI of 20–30 units) with all the lysates regardless of activation status of cells.

Phosphorylation of B-cell Signaling Proteins in Ramos Cells—A multiplex immunoassay was evaluated for detecting phosphorylation of multiple signaling proteins in the well-characterized Ramos B-cell line, which was derived from an individual with Burkitt lymphoma. The same antibody-coated microbead sets described in Fig. 1 for the analysis of signaling in Jurkat T-cells were used (Fig. 1). As shown in Fig. 3B, Syk was more strongly phosphorylated in Ramos cells than its homologue Zap-70 was in Jurkat cells (Fig. 1) after the treatment with sodium pervanadate. This result is consistent with Syk being a much more active kinase than Zap-70 (37). Specificity of the anti-Syk antibody coated on microbeads was demonstrated by detection of Syk in lysates from the B-cell lines by IP/WB analysis (Fig. 4). Importantly as mentioned above, this antibody was not reactive in IP/WB analysis performed on Jurkat (E6-1) T-cell lysates (Fig. 1B). In contrast to the multiplex microbead immunoassay analysis of Jurkat T-cell activation, phosphorylation of CD3 was undetectable, whereas phosphorylation of Zap-70 was marginal in Ramos B-cells (Fig. 3). IP/WB analysis was consistent with this observation, showing low level expression of Zap-70 protein and its phosphorylation in Ramos B-cells (Fig. 4). Taken together, results of the multiplex analysis presented in Figs. 1A and 3 and IP/WB data presented in Figs. 1B and 4 demonstrate utility of the multiplex microbead analysis for T-cell and B-cell signaling pathways.

Expression of Signaling Proteins in B-CLL Cell Lines—The multiplex analysis of B-cell signaling was extended to cell lines derived from patients with CLL. First the conventional methods of IP/WB were used to assess total and phospho-
rulated proteins (Syk, Zap-70, Lck, and LAT) in CLL B-cell lines (Mec-1 and MO1043) to make comparisons with Ramos B-cells. Both of the Mec-1 and MO1043 lines expressed Syk protein (Fig. 4). The data also showed that Ramos and Mec-1 cells had low levels of Zap-70. In contrast, the MO1043 cells expressed a high amount of Zap-70 (Fig. 4). Thus, the MO1043 cell line may be representative of B-CLL cases where patient leukemic cells have strong expression of Zap-70 protein (38, 39). Normal, mature B-cells, except certain subpopulations, do not express Zap-70 (40). Two other T-cell proteins, Lck and LAT, not usually expressed by mature B-cells (20, 41), were also examined. Ramos cells produced a relatively high amount of Lck, and Mec-1 and MO1043 cells both showed barely detectable amounts of Lck (Fig. 4). In addition, Ramos B-cells displayed low level expression of LAT, whereas LAT was undetectable in Mec-1 and MO1043 cells by IP/WB analysis (Fig. 4). Analysis of tyrosine phosphorylation of Syk, Zap-70, Lck, and LAT with and without sodium pervanadate treatment was also performed, and the data are shown in Fig. 4 (left panel for each protein). In general, where protein expression was detectable, phosphorylation of the protein in treated cells was also detectable, i.e. the phosphorylation level was proportional to the level of expression. For example, Lck and LAT were expressed and phosphorylated in Ramos B-cells. Expression of these proteins was low to none in Mec-1 and MO1043 cells; accordingly phosphorylation of LAT in both of these cell lines was also not detectable (Fig. 4).

Surprisingly Zap-70 expression in MO1043 cells did not follow this pattern. Although Zap-70 protein was expressed in high amounts in MO1043 cells, the phosphorylation of Zap-70 in this cell line after treatment with sodium pervanadate was not strong (Fig. 4).

**Multiplex Suspension Array Analysis of B-cell Signaling in CLL Cell Lines**—In general, B-CLL cells not only have a low surface expression of BCR but also respond poorly to activation by anti-IgM antibodies (42). Detection of surface expression of BCR in the CLL cell lines, in comparison with the Ramos B-cell line (Burkitt lymphoma), was performed by flow cytometric analysis using anti-IgM antibody. This analysis revealed that Ramos cells expressed high levels of surface IgM (data not shown). In contrast, all three CLL cell lines expressed low levels of surface IgM (data not shown) as originally reported (29, 30). Accordingly treatment with anti-IgM antibody resulted in strong phosphorylation of Syk in Ramos cells, whereas no detection of Syk phosphorylation was observed by IP/WB analysis in the three CLL cell lines (Mec-1, Mec-2, and MO1043) treated with anti-IgM antibody (data not shown). Therefore, for experimental consistency in the phosphorylation of various signaling proteins in the Ramos cells and these three CLL cell lines, the cells were treated with sodium pervanadate.

For multiplex microbead suspension array analysis of CLL cell lines, lysates from sodium pervanadate-treated and untreated Mec-1, Mec-2, and MO1043 cells were tested for
phosphorylation of several cell signaling proteins. Phospho-
proteomic profiles of signaling proteins in two of the cell lines, Mec-1 and MO1043, are shown in Fig. 6. The same capture antibody microbead sets used in Figs. 1 and 3 were used for this analysis. Table I shows the relative -fold activation of Zap-70, Syk, and other signaling proteins in the Mec-1 and MO1043 lines as well as the Mec-2 line. For the receptor-proximal signaling events, the -fold activation of Syk in the CLL lines was comparable or higher than that in Ramos B-cells with the notable exception of MO1043 cells. Syk activation was dramatically reduced in MO1043 cells as compared with the other CLL cell lines (Table I). Similarly despite strong expression of Zap-70 in MO1043 cells, the -fold activation of Zap-70 in this cell line was much lower than that of Jurkat T-cells (81% lower, Table I). This lack of Zap-70 activation could be due to the effects of Epstein-Barr virus transformation and/or mutation in the zap-70 gene. Mec-2 cells exhibited significantly higher Syk activation compared with Ramos and Mec-1 cells, both of which displayed comparable Syk activation (Table I). Both Mec-1 and Mec-2 cell lines were established from the same patient’s blood, drawn within approximately a 1-year interval (29). The Mec-2 cell line was established near the terminal stage of CLL disease when the patient’s white blood cell count was more than 3-fold higher compared with the time when the Mec-1 cell line was established (29). Thus, the significantly higher Syk activity in the Mec-2 cell line may correlate with the advanced stage of malignancy.

To assess cytosolic signaling events in the CLL cell lines, the -fold increase in activation of Akt, Erk, and Rsk was examined. The data are shown in Fig. 6 and Table I. The most dramatic difference was observed for Akt activation. As in the case of Syk, -fold activation of Akt was dramatically increased in Mec-2 cells as compared with Mec-1 and Ramos cells. The activity of transcription factors CREB and STAT3 was also examined. In the MO1043 cell line, which expressed a high amount of Zap-70, activation of CREB was severely inhibited (Fig. 6 and Table I).

**DISCUSSION**

This report describes a novel multiplex suspension array approach to study cell signaling pathways by simultaneous analysis of phosphorylation of multiple signaling proteins in each sample. As a model signaling pathway, we examined lymphoid cell activation in the Jurkat (E6-1) T-cell tumor line (26). In addition, the multiplex phosphorylation immunoassay was extended to investigate B-cell activation in a Burkitt lymphoma cell line (Ramos) and three B-CLL cell lines. We demonstrate that the microbead suspension array immunoassay is versatile and sensitive. Phosphoproteomic profiling of 10 signaling proteins (CD3, Lck, Zap-70, LAT, Erk, Akt, Rsk, CREB, STAT3, and Syk) in the Jurkat T-cell line needed ~8 × 10⁴ cells (Fig. 1A). In contrast, to obtain the same amount of information by IP/WB and ELISA analyses of all 10 signaling proteins, approximately 500 times and 100 times more Jurkat cells (4 × 10⁷ and 8 × 10⁸), respectively, were required (Fig. 1B; ELISA data not shown). Thus, the need for only a small amount of sample for the phosphoproteomic profiling of several signaling proteins (theoretically up to 100) is a clear advantage. Additionally this assay format readily enables the use of internal controls. In comparison with fixed formats such as peptide array systems (43), the microbead suspension array approach described here offers flexibility and ease of adaptability where microbead sets coated with capture antibodies can be included or excluded from the mixture at will. Methods based on two-dimensional gel electrophoresis and MS have been applied recently for simultaneous analysis of multiple signaling proteins in cells (44–48). However, these methods require complex protocols as well as complicated and very costly instrumentation for broad biological and clinical applications. Nevertheless these methodologies have an advantage over immunoassays as they do not depend upon the availability of highly specific and high affinity antibodies. In contrast, development of the microbead suspension array immunoassay described in this report, as for other more commonly used immunoassays (e.g. immunoprecipitation, ELISA, and planar antibody array), required well characterized antibodies with high specificity and affinity. This technology has a dynamic range of over a thousandfold; therefore groups of analytes, e.g. cytokines/chemokines (17), that are found in concentration ranges outside of the dynamic range can be efficiently measured in panels of several dozen analytes in the multiplex format. Also in the context of signaling pathways, for the global proteomic applications, the two-dimensional gel electrophoresis/MS methodologies may be more suitable, whereas the multiplex microbead suspension array immunoassay enables more targeted analysis of specific components of signaling pathways.

For the multiplex microbead suspension array approach,
monoclonal antibodies to different signaling proteins were conjugated to individually identifiable microbead sets to serve as capture molecules. Results of the multiplex microbead immunoassay using these capture antibodies were confirmed by IP and WB analysis (Figs. 1, A and B, and 4). Importantly as a key feature of the multiplex immunoassay for detection of tyrosine phosphorylation of several signaling proteins (CD3, Lck, Zap-70, LAT, and Syk), the single detection reagent, anti-phosphotyrosine antibody (i.e. the 4G10 antibody), was accurate and sufficient. This procedure circumvents the need for mixtures of phosphotyrosine protein-specific antibodies; such mixtures may require additional optimization of the multiplex assay to minimize reagent cross-reactivity. However, detection of phosphorylation of serine/threonine residues in the relevant signaling proteins was performed by using phosphoprotein-specific antibodies. The data (Figs. 1, A and B, and 3–5) clearly show the similarity in the extent of phosphorylation of each signaling protein in untreated and pervanadate-treated cells as detected by multiplex method and the IP/WB method. Importantly specificity of multiplex suspension array format was well demonstrated by the phosphorylation analysis of two kinases, Zap-70 and Syk. Zap-70 is expressed in T-cells and usually not in mature B-cells, whereas Syk is expressed in mature B-cells. In this study, Zap-70 phosphorylation was detected by the multiplex phosphorylation assay in Jurkat (E6-1) T-cells. A low level of Zap-70 phosphorylation was detected in Ramos B-cells by the multiplex assay (Fig. 3). This result is consistent with the low level of expression and phosphorylation of Zap-70 detected by IP/WB (Fig. 4). In contrast to Zap-70, strong phosphorylation of Syk was detected in Ramos B-cells by both the multiplex assay (Fig. 3) and IP/WB (Fig. 4) and not in Jurkat (E6-1) cells (Fig. 1, A and B). However, as mentioned under “Results,” pervanadate treatment of cells leads to general phosphorylation of most of the intracellular tyrosine kinases and their substrates and is therefore not specific to a given pathway (32, 33). Targeted stimulation of the T-cell activation pathway was achieved by anti-CD3 antibody as described below. Nevertheless the above results clearly demonstrate specificity of the multiplex assay for the detection of Zap-70 and Syk phosphorylation. Phosphoproteomic profiles of differentially expressed and activated Zap-70 and Syk in various populations of T-cells and B-cells (9, 40, 49) can therefore be efficiently and accurately studied with the microbead suspension array immunoassay described here.

The utility of this novel multiplex phosphorylation assay was further demonstrated by the analysis of various signaling proteins in a time course activation of Jurkat (E6–1) cells treated with anti-CD3 antibody, which activates the T-cell signaling pathway (Fig. 2). This analysis highlighted the following features of the multiplex assay. (a) Activation kinetics of Lck and Zap-70 were similar to those observed in the immunological synapse between T-cell and antigen-presenting cell using immunofluorescence and confocal microscopy (36). (b) The assay allowed investigation of temporal changes in phosphorylation of several proteins simultaneously in a kinetic experiment involving multiple time points. (c) The assay was convenient for monitoring the peak activation level as well as signal decay of multiple signaling proteins simultaneously.

Analysis of Syk phosphorylation in three B-cell lines (Ramos, Mec-1, and MO1043), enabled direct comparison of Syk activation between these cell lines (Fig. 5). In addition, phosphorylation levels of different signaling proteins in stimulated cells, as indicated by MFI values, were compared with the constitutive levels of phosphorylation in non-stimulated cells. These comparisons allowed assessment of fold activation of Syk in each cell line. Activation of Syk and other signaling proteins in all of the B-cell lines in this study are presented in Table I. Because the multiplex microbead suspension array immunoassay described in this report is easily adaptable to a high throughput format, a large number of samples can be analyzed, and activities of multiple signaling proteins can be determined simultaneously. In contrast, conventional approaches such as IP and WB would not be practical for high throughput analysis.

The study of cell signaling components in B-CLL cell lines by the microbead suspension array immunoassay has revealed potential implications for the biology of CLL as described below. As shown by the IP/WB analysis, the MO1043 CLL cell line expressed a high level of Zap-70 protein (Fig. 4). High expression of Zap-70 in leukemic cells from B-CLL patients correlates with a more aggressive disease (27, 28). In contrast, a very low level of Zap-70 expression was observed in Mec-1 (Fig. 5) and Mec-2 (data not shown) cell lines. Noteworthy are the following observations from the multiplex phosphoproteomic analysis. (a) The activity of Syk was severely inhibited in MO1043 cells (44% of that observed for Ramos B-cells). (b) Despite strong expression of Zap-70 in MO1043 cells (Fig. 4), fold activation of Zap-70 was only about 19% of that observed in Jurkat cells (Table I). These
data, however, are not in agreement with previous studies showing that Syk phosphorylation was enhanced in primary CLL tumor cells in association with Zap-70 expression (39, 50). Thus, it is possible that a defect in the anomalously expressed Zap-70 protein in MO1043 B-CLL cells may have resulted in reduction of the activities of Syk and Zap-70 proteins observed in our study.

A clinical hallmark of CLL is the accumulation of leukemic cells over several years with an apparent defect in apoptosis (29, 51). It is also noteworthy that in our study the activity of Akt, a key signaling component that promotes cell survival (52), was substantially higher in two of the CLL cell lines, Mec-2 and MO1043, in comparison with the Mec-1 cell line (Fig. 6 and Table I). MO1043 in addition displayed very low activation of CREB (4% of that in Ramos cells, Fig. 6 and Table I). Low CREB activity may enhance bcl-2 gene expression resulting in the increase in cell survival (53). The Mec-1 and Mec-2 lines are particularly interesting as they were established from the same CLL patient with the following differences. (a) The Mec-1 line was derived more than a year before the patient succumbed to disease (white cell count, 39 × 10^3 cells/ml). (b) The Mec-2 line was derived near the terminal stage of disease (white blood cell count, 131 × 10^3 cells/ml) (29). The -fold activation of Akt was dramatically higher in Mec-2 cells compared with Mec-1 cells (Table I). This finding suggests that increased activity of Akt may represent a molecular mechanism responsible for the accumulation of leukemic cells by increased antiapoptotic signaling. Analysis of primary CLL cells isolated from a number of B-CLL patients will be needed to establish the biological relevance of our observations, made by multiplex microbead suspension array analysis, on the relative activities of various signaling proteins in CLL cell lines.

Taken together, the data presented in this report demonstrate the utility of multiplex microbead suspension array format for the analysis of multiple components of intracellular signaling pathways. Methods for simultaneous analysis of activities of large numbers of signaling proteins will be essential for basic research objectives that aim to define and integrate molecular mechanisms that regulate normal cell function. Multiplex phosphoproteomic analysis will facilitate translational research on biomarkers by developing profiles of cell signaling proteins; such profiles can be used for improved diagnosis and prognosis of a variety of diseases (47, 54). In addition, as more targeted therapeutic agents make their way into the clinic, efficient and reliable multiplex methods will be critical for rapidly and comprehensively assessing cellular signaling events and pathways impacted by such therapies (3, 55).

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