Quantitative Proteomic and Transcriptional Analysis of the Response to the p38 Mitogen-activated Protein Kinase Inhibitor SB203580 in Transformed Follicular Lymphoma Cells*†§

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The p38 mitogen-activated protein kinase (MAPK) is a key mediator of stress, extracellular-, growth factor-, and cytokine-induced signaling, and has been implicated in the development of cancer. Our previous work showed evidence for p38 MAPK activation in a subset of transformed follicular lymphomas (Elenitoba-Johnson et al. 2003 *Proc. Natl. Acad. Sci. U.S.A.* 100, 7259). We demonstrated that inhibition of p38 MAPK by SB203580 resulted in dose- and time-dependent caspase-3-mediated apoptosis. In order to further elucidate the basis of the cellular effects of SB203580, we have employed a systems biologic approach involving cDNA microarray and quantitative proteomic analysis of transformed follicular lymphoma derived-cells (OCI Ly-1) treated with SB203580. Gene expression profiling revealed differential expression (≥1.5-fold) of 374 genes/ESTs in cells treated for 3 h and 515 genes/ESTs in cells treated for 21 h. The majority (52% at 3 h and 91% at 21 h) were down-regulated, including genes encoding growth cytokinins, transcriptional regulators, and cytoskeletal proteins. Quantitative proteomic analysis using ICAT-LC-MS/MS identified 277 differentially expressed proteins at 3 h and 350 proteins at 21 h of treatment with SB203580, the majority of which were also down-regulated. Analysis of functional groups of the differentially expressed proteins implicated components of diverse overlapping pathways including the IL-6 phosphatidylinositol 3-kinase, insulin-like growth factor 2/Ras/Raf, WNT8d/Frizzled, MAPK-activated protein kinase 2, and nuclear factor κB. The differential phosphorylation status of selected kinase-active proteins was validated by Western blotting analysis. Our complementary genomic and proteomic approach reveal the global cellular consequences of SB203580 treatment and provide insights into its growth inhibitory effect on transformed follicular lymphoma cells. *Molecular & Cellular Proteomics* 3:820–833, 2004.

An important aim of functional genomic research is to globally identify and quantify specific proteomic and/or transcriptional changes that are associated with physiologic or diseased states, thus enabling the elucidation of genes/proteins and signaling pathways that are associated with the phenotypic expression of a particular cellular state. The currently available microarray technologies permit the quantification of tens of thousands of gene transcripts and have been largely successful in demonstrating the global transcriptional changes accompanying the transition between cellular quiescence and activation, or during transition from normal to pathologic states (1–7). Proteomics, on the other hand, allows identification and quantification of up to a few thousand proteins, limited by the currently available technologies and the complex nature of the proteome especially in higher eukaryotes and mammalian cell systems. While the most widely used proteomics approach is the two-dimensional (2-D)† gel-based method followed by MS (8–10), the recent development of multidimensional liquid chromatographic methods combined with MS/MS (LC-LC-MS/MS) has permitted sensitive detection of low-abundance proteins, membrane proteins and proteins with extreme pI (11–15). The ability to perform global quantitative proteomics has been significantly enhanced by the advent of the ICAT-based technology that is efficient in simplifying the proteome, and in combination with three-dimensional 3-D LC-MS/MS permits detection and quantifi-

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*The abbreviations used are: 2-D, two-dimensional; LC-LC, multidimensional chromatography; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; STAT4, signal transducer and activator of transcription 4; MAPKAPK2, mitogen-activated protein kinase activated protein kinase 2; NFAT, nuclear factor of activated T cells; FL, follicular lymphoma; TGF-β, transforming growth factor beta; IL-6, interleukin-6; FGF6, fibroblast growth factor 6; IGF2, insulin-like growth factor 2; TNF, tumor necrosis factor; uPA, urokinase plasminogen activator; MMP, matrix metalloproteinase; PKB, protein kinase B; PI3K, phosphatidylinositol 3-kinase; Hsp90, heat shock protein 90; EST, expressed sequence tag; 3-D, three-dimensional; NF-κB, nuclear factor κB; M KK, MAPK kinase; JNK, c-Jun N-terminal kinase.

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Proteomic and Transcriptional Consequences Associated with SB203580 Treatment

cation of proteins and peptides from very complex samples (11, 16, 17).

p38 mitogen-activated protein kinase (MAPK) is a member of the MAPK superfamily. Four structurally homologous isoforms of p38 MAPK, p38α, p38β, p38γ, and p38δ, have been identified (18–23). Activation of p38 MAPK is commonly induced by stress stimuli, proinflammatory cytokines, and growth factors (24–27) and is mediated by its direct upstream effectors MAPK kinases (MAPKKK4/6) via phosphorylation of the TGY motif (18, 28, 29). Activation of p38 MAPK leads to phosphorylation and activation of signal transducer and activator of transcription 4 (STAT4), MAPK-activating protein kinase 2 (MAPKAPK2), nuclear factor of activated T cells (NFAT), nuclear factor-κB (NF-κB), and activating transcription factor-6, resulting in diverse cellular responses including cell invasiveness, differentiation, growth, and transformation (30–35). A well-characterized role of p38 MAPK is the mediation of apoptotic and anti-proliferation signals (36–38); however, p38 MAPK also conveys growth promoting and anti-apoptotic signals (39–41). Recently, p38 MAPK has been identified as an important mediator of proliferation and transformation signals of cytokines and growth factors in prostate cancer (40), breast cancer (41), and lymphoma/leukemia cell lines (42). p38 MAPK also mediates production of proliferative cytokines and growth factors induced by other growth stimuli in various types of cells including B and T lymphocytes (43–49).

Our previous work showed that p38 MAPK was up-regulated and constitutively activated during the transformation of follicular lymphomas, one of the most common low-grade B cell malignancies, to the aggressive diffuse large B cell lymphoma (1). In addition, selective inhibition of p38 MAPK by a widely used pyridinyl imidazole compound SB203580 (50–54) resulted in a dose- and time-dependent growth inhibition and apoptosis in transformed follicular lymphoma (FL) cells that overexpress p38 MAPK. These data indicate that induction and activation of p38 MAPK is associated with the pathogenesis and progression of a subset of B cell lymphomas. However, very little is known as to the sequelae of deregulated p38 MAPK signaling in B cell lymphoma, or what the global cellular effectors of SB203580-mediated growth inhibition are in transformed lymphoma cells.

In order to further elucidate the cellular consequences of SB203580 exposure in transformed FL, we employed a functional genomic approach involving both quantitative proteomic analysis and transcriptional profiling of transformed follicular lymphoma-derived cells (OCI Ly-1) treated with SB203580. Both cDNA microarray and proteomic analysis identified several hundred differentially expressed transcripts or proteins following exposure of the lymphoma cells to SB203580. In particular, our studies revealed the induction of proapoptotic genes at transcriptional and translational levels upon lymphoma cell exposure to SB203580. Conversely, growth, anti-apoptosis, and tumor invasion genes/proteins were repressed. Importantly, we identified several differentially regulated genes and proteins that have not been previously associated with p38 MAPK signaling in B-lineage lymphoma cells and represent novel pathways that are affected by SB203580. Our studies highlight the complementary nature of gene expression profiling and proteomic analysis in the elucidation of the cellular response to growth inhibition effected by a specific compound and the interconnected pathways involved in such complex processes.

EXPERIMENTAL PROCEDURES

Cell Cultures, Drug Treatment, and MTT Assay—OCI Ly-1 cells were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% heat-inactivated FCS, 2 mm L-glutamine, and 100 U/ml of penicillin-streptomycin mixture (Life Technologies, Inc.) at 37 °C and 5% CO₂. Unless specified otherwise, SB203580 (Calbiochem, San Diego, CA) was dissolved in DMSO and added to cells (final concentration 40 μM) cultured at a starting density of 2.0 × 10⁶ cells/ml. Control cells were treated with equivalent volume of DMSO. Cell viability was determined using the MTT assay as described previously (55).

Cell Lysates and Immunoblot Analysis—Cells were lysed using the following buffer: 100 mM Tris-HCl, pH 8.5, containing 0.1% SDS and 8 μM urea (Sigma, St. Louis, MO). Protein concentrations of cell extracts were measured using the Bradford protein assay kit (Pierce, Rockford, IL). For immunoblot analysis of proteins, up to 50 μg proteins per lane were resolved in a 12% SDS-PAGE gel using a Bio-Rad mini gel system. Separated proteins were subsequently transferred onto a nitrocellulose film using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA). The following antibodies were used for immunoblot analysis: rabbit polyclonal antibodies against IL-6, fibroblast growth factor (FGF)-6, Raf-1, and Frizzled2 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal antibody against insulin-like growth factor 2 (IGF-2; Upstate, Charlottesville, VA), and rabbit polyclonal antibodies against phospho-Raf-1, NF-κB, phospho-NF-κB, MAPKAPK2, phospho-MAPKAPK2, MAPK kinase 4 (MKK4), phospho-MKK4, c-Jun N-terminal kinase 3 (JNK3), and phospho-JNK3 (Cell Signaling, www.cellsignal.com). Immunoreactive protein bands were visualized using the ECL kit (Amersham Pharmacia Biotechology Inc.). Expression ratios between control and treated samples were normalized to the loading control.

Gene Expression Profiling—Total RNA was extracted using the TRIzol reagent (Life Technologies, Carlsbad, CA). Messenger RNA was purified from total RNA using poly-T coated beads (Oligotex mRNA Isolation Kit; Qiagen, Valencia, CA), according to the manufacturer’s protocol. cDNA microarray analysis was performed as previously described (1) with the following modification: the 9,600-clonset set (Research Genetics, Huntsville, AL) was used for a direct two-color hybridization (Cy5 = treated samples versus Cy3 = control sample). Reproducibility of gene expression ratios across four replicates was accomplished by using GENESPRING™ software (Silicon Genetics, Redwood City, CA). To ensure that the reported fold changes in gene expression were significant, p < 0.05 was used as a cut-off value so that only those fold changes with p values less than 0.05 were presented.

ICAT Labeling of Proteins and 3-D LC-MS/MS—ICAT labeling and 3-D LC were performed as described previously (11, 12) with some modifications (Fig. 1). Equal amounts of protein (1 mg) in 800 μl of lysis buffer were taken for each sample (control or treated). Proteins were reduced with 2.5 mM Tris [2-carboxyethyl] phosphate (Pierce) for
30 min at 37°C. ICAT labeling was carried out by mixing 10 U of light (for control samples) or heavy (for treated samples) cleavable ICAT (Applied Biosystems, Foster City, CA) with respective protein samples following manufacturer's instruction and incubated for 90 min at 37°C. Labeled proteins from control and treated samples were mixed and dialyzed against 250 ml of urea buffer (2 M urea, 10 mM Tris, pH 8.5) three times for 1 h each at room temperature using the 3.5 kDa cut-off dialysis cassette (Pierce). The dialyzed protein mixtures were then diluted 2-fold with 10 mM Tris, pH 8.5, before digestion with 20 µg of modified trypsin (Promega, Madison, WI) overnight at 37°C. The peptide mixture was then acidified to pH 3.0 by adding TFA before loading onto the cation exchange column. The 3-D liquid chromatographic separation of peptides was carried out following these three steps. First, strong cation exchange chromatography using a 3.6-mm × 20-cm polysulfoethyl A column (Poly LC Inc., Columbia, MD) at a flow rate of 400 µl/min with 45 fractions collected (600 µl/fraction). A two-step linear buffer gradient was used: 5% buffer B and 95% buffer A to 25% buffer B and 75% buffer A for 50 min followed by 25% buffer B and 75% buffer A to 100% buffer B for 18 min (buffer A, 20 mM KH₂PO₄, 25% ACN, pH 3.0; buffer B, 350 mM KCl in buffer A, pH 3.0). Second, each fraction from the strong cation exchange chromatography was further purified by a ultralink monomeric avidin affinity cartridge (Applied Biosystems) to enrich only the cysteine-containing and ICAT-labeled peptides following the manufacturer's instructions. The eluted peptides were then dried and cleaved as instructed. Third, reverse-phase capillary chromatography was performed using an in-house packed C18 column (100 µm × 10 cm; Michrom BioResources, Inc., Auburn, CA) and an ACN gradient (12). The peptide samples were loaded and analyzed by a Surveyor LC linked to a LCQ DECA XP ion trap tandem mass spectrometer (ThermoFinnigan, San Jose, CA) using an automated method as previously described (56).

**Protein Identification and Quantification**—All MS/MS spectra were searched against the human protein database established by National Center for Biotechnology Information (NCBI) using the SEQUEST™ algorithm (15). Stable modification of cysteine was set as 227 and differential modification of cysteine set as 9. Proteins were identified based on the scoring criteria described previously (11) with the following modification: only identifications that fulfilled the following criteria were included in the search results, peptides of single, double, and triple charges should have a respective cross correlation score (Xcor) ≥ 2.0, 2.0, and 3.0. Probability of identified proteins was analyzed using ProteinProphet™ (57). Protein quantification was performed using the XPRESS™ software (ThermoFinnigan), which automatically calculates the relative abundance of light and heavy ICAT-labeled peptide as a ratio of light versus heavy (16).

**RESULTS**

Inhibition of p38 MAPK by SB203580—Inhibition of p38 MAPK by SB203580 in transformed FL cells expressing ki-
Proteomic and Transcriptional Consequences Associated with SB203580 Treatment

Kagawa et al.

nase-active p38 MAPK resulted in growth inhibition and apoptosis (1). To further study the p38 MAPK signaling pathways involving FL transformation, we utilized a t(14;18)-positive follicular lymphoma-derived cell line (OCI Ly-1). These cells displayed a dose- and time-dependent growth inhibition in response to SB203580 as shown in MTT assays (Fig. 2A). Furthermore, the level of kinase-active p38 MAPK constitutively expressed in these cells was significantly decreased by SB203580 after 3 h of treatment (Fig. 2B), demonstrating that SB203580 inhibited p38 MAPK.

**Differential Transcriptional and Proteomic Analysis of SB203580-treated Cells**—SB203580 treatment of OCI Ly-1 cells resulted in down-regulation of several hundred genes and ESTs after 3 h (Table I). Of the 9,600 genes or ESTs studied, 374 (3.9%) were differentially expressed (1.5-fold up- or down-regulated) at 3 h and 515 (5.4%) were differentially expressed at 21 h of SB203580 treatment. Up to 52% of the differentially expressed genes and ESTs were down-regulated at 3 h of treatment, while 91% of the differentially expressed genes and ESTs were down-regulated at 21 h of treatment.

Quantitative proteomic analysis of OCI Ly-1 cells treated with SB203580 identified over 2,500 unique peptides from each experiment at 3- and 21-h treatment time points (Table II). One-third of these peptides were ICAT-labeled cysteine-containing peptides. Identification of a peptide was made based on the m/z ratio (Fig. 3A) followed by MS/MS (Fig. 3B) of the peptide ion. Probability of identified proteins calculated by ProteinProphet™ showed that majority of the proteins identified were true positive identification. Over 89% of identified proteins had a $p_{comp} \geq 0.8$ with an error rate $\leq 1.7$%; over 94% of the identified proteins had a $p_{comp} \geq 0.5$ with an error rate $\leq 7.3$% (Fig. 4). Expression ratio of each protein was automatically calculated by the Xpress™ software based on the relative abundance between light ICAT (Fig. 3C, a) and heavy ICAT (Fig. 3C, b)-labeled peptides. Distribution of expression ratios of all quantified proteins showed a spectrum of normal ratio distribution (Fig. 5). Of the peptides that were ICAT labeled, 277 differentially expressed proteins (1.5-fold up- or down-regulated) were identified at 3 h and 350 differentially expressed proteins at 21 h of treatment (Table II). The majority of these differentially expressed proteins were down-regulated (82% at 3 h and 76% at 21 h) by SB203580. The differentially expressed proteins account for ~35.3% of all proteins quantified at 3 h and 37.6% of total quantified proteins at 21 h of SB203580 treatment when the threshold of differential expression was set at 1.5-fold. At the 2.0-fold threshold, about 21.5% of quantified proteins for 3-h treatment and 22.1% for 21-h treatment were differentially expressed. All functional analyses of differentially expressed proteins were performed using the 1.5-fold threshold. Among all proteins identified and quantified, ~90% are proteins with known functions while 10% are hypothetical proteins. The general pattern of proteomic and transcriptional profiling indicated that many cellular activities were down-regulated upon inhibition of p38 MAPK by SB203580.

**Functional Groups of Differentially Expressed Genes and Proteins**—To further analyze the functions of differentially regulated genes and proteins, we performed a PubMed literature search (www.ncbi.nlm.nih.gov/ PubMed) for each gene by name or NCBI protein function summary (www.ncbi.nlm.nih.gov/protein) using the protein accession number. We then assigned each gene or protein into one of 13 functional groups such as growth, apoptosis, cytoskeleton, transcriptional regulator, and hypothetical proteins based on the search result (see Supplemental Tables 1–4 for transcript analysis and Supplemental Tables 5–8 for protein analysis). Summaries of the functional groups of genes and proteins are shown in Tables I and III. In detail, we identified a substantial number of differentially expressed genes and ESTs (Table I) with diverse functions. Most growth-associated genes were underexpressed in SB203580-treated cells, with 19 out of 25

### Table I

**Summary of up- or down-regulated transcripts (≥1.5-fold)*

| Functional groups                  | Non-treated vs treated (3 h) | Non-treated vs treated (21 h) |
|------------------------------------|-----------------------------|-------------------------------|
|                                    | Up  | Down | Up  | Down |
| Growth-related                     | 6   | 19   | 2   | 46   |
| Anti-growth                        | 5   | 0    | 1   | 2    |
| Pro-apoptosis                      | 3   | 3    | 0   | 2    |
| Anti-apoptosis                     | 3   | 0    | 0   | 4    |
| Biosynthesis                       | 1   | 1    | 0   | 6    |
| Cytoskeleton                       | 9   | 2    | 4   | 4    |
| Tumor invasion/cell mobility       | 3   | 4    | 0   | 5    |
| Transcription/translation regulator| 7   | 7    | 1   | 11   |
| Other signaling                    | 15  | 20   | 3   | 38   |
| Chaperone                          | 0   | 0    | 0   | 1    |
| Extracellular matrix               | 2   | 2    | 1   | 2    |
| Hypothetical                       | 13  | 10   | 3   | 25   |
| ESTs and others                    | 114 | 128  | 32  | 322  |
| Total                              | 178 | 196  | 47  | 468  |

* The numbers represent transcripts that are up- or down-regulated (≥1.5-fold) in each functional group.

### Table II

**Summary of peptides and proteins identified by 3-D LC-MS/MS in OCI LY-1 cells treated with SB203580**

|                  | No. of unique peptides | No. of identified proteins | No. of ICAT-labeled C-peptidesa |
|------------------|------------------------|----------------------------|--------------------------------|
| Nontreated vs treated (3 h) | 2,501                  | 1,121                      | 783                            |
| Nontreated vs treated (21 h) | 2,599                  | 1,297                      | 930                            |

|                  | Up-regulated | Down-regulated |
|------------------|--------------|----------------|
| Nontreated vs treated (3 h) | 49           | 228            |
| Nontreated vs treated (21 h) | 84           | 266            |

a The ICAT-labeled cysteine-containing peptides enable relative quantification of the peptides and proteins.
and 46 out of 48 genes down-regulated at 3 and 21 h of treatment, respectively. Among the down-regulated genes, several cyclins, cyclin-dependent kinases, Ras proteins, thrombospondin 1 and 2, and transforming growth factor-β (TGF-β) were detected at both time points of SB203580 treatment (see Supplemental Tables 1 and 2). Several biosynthesis associated genes (ribosomal protein-coding genes), transcriptional regulators (c-fos, IκB, Max, GATA binding protein 4, and p53 binding protein 1) were also underexpressed following drug treatment (see Supplemental Tables 1 and 2).

Analysis of functional groups of differentially expressed proteins also identified substantial numbers of up- or down-regulated proteins with diverse functions, including growth-associated proteins (37 at 3 h and 36 at 21 h), transcription/translation regulating proteins (24 at 3 h and 46 at 21 h), signaling proteins involved in development and differentiation (21 at 3 h and 26 at 21 h), biosynthetic proteins (12 at 3 h and 10 at 21 h), and hypothetical proteins (27 at 3 h and 34 at 21 h) (Table III). The great majority (>90%) of growth associated

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**Fig. 3.** Full MS scan (A), tandem MS scan (B), and quantification (C) of a peptide. A, arrow points to the peptide ion selected for fragmentation. B, tandem MS spectrum matches a light-ICAT labeled triply charged peptide. C, relative abundance of light ICAT-labeled (a, control) and heavy ICAT-labeled (b, treated sample) peptide automatically calculated by the Xpress™ software.

**Fig. 4.** Protein identification error and sensitivity rates determined by ProteinProphet™. A, analysis of proteins identified at 3 h of SB203580 treatment. B, analysis of proteins identified at 21 h of SB203580 treatment. Error (%) represents the portion of identified proteins that were false positive, while sensitivity (%) shows the portion of identified proteins at a given \( p_{\text{comp}} \) threshold.
proteins such as IL-6, FGF6, IGF2, and several WNT proteins were down-regulated at both 3 and 21 h of drug treatment (Table IV). Most of the ribosomal proteins (grouped as biosynthetic proteins), motility and invasion-related proteins (matrix metalloproteinase (MMP)-12, urokinase plasminogen activator (uPA) receptor (uPAR), and cathepsin V), protein chaperones (heat shock protein (Hsp) 60, Hsp70, and Hsp90), extracellular matrix proteins (collagen, usherin, fibrillins, and laminin), and transcription-regulating proteins (MAPKAPK2, NF-κB, IκB, STAT4, v-Myc, ONECUT 2, and REST) were also down-regulated upon treatment of SB203580.

Over a hundred proteins were listed as miscellaneous proteins. These are proteins that may play roles other than the 12 listed groups including a large number of metabolic enzymes such as NADH2 dehydrogenase and ATPase. Although concordance between microarray and proteomic analysis was low (~20%) (Supplemental Tables 1–8), functional analysis of the differentially expressed genes and proteins showed a similar pattern of changes, especially those involved in growth regulation and gene transcription.

**Proteomics and Transcriptional Consequences Associated with SB203580 Treatment**

### Table III

| Functional groups                  | Nontreated vs treated (3 h) | Nontreated vs treated (21 h) |
|------------------------------------|-----------------------------|------------------------------|
|                                    | Up  | Down | Up  | Down |
| Growth-related                     | 1   | 36   | 3   | 33   |
| Anti-growth                        | 3   | 0    | 2   | 0    |
| Pro-apoptosis                      | 2   | 0    | 4   | 0    |
| Anti-apoptosis                     | 0   | 3    | 0   | 1    |
| Biosynthesis                       | 0   | 12   | 1   | 9    |
| Cytoskeleton                       | 3   | 4    | 3   | 7    |
| Tumor invasion/cell mobility       | 0   | 5    | 0   | 6    |
| Transcription/translation regulator| 4   | 20   | 12  | 34   |
| Other signaling proteins           | 4   | 17   | 7   | 19   |
| Chaperone proteins                 | 0   | 5    | 0   | 5    |
| Extracellular matrix               | 0   | 3    | 0   | 6    |
| Hypothetical proteins              | 0   | 5    | 0   | 5    |
| Miscellaneous                      | 9   | 18   | 8   | 26   |
|                                   | 21  | 80   | 39  | 88   |

*The numbers represent proteins that are up- or down-regulated (≥1.5-fold) in each functional group.*

of proteins shown in Table IV. Most of the ribosomal proteins (grouped as biosynthetic proteins), motility and invasion-related proteins (matrix metalloproteinase (MMP)-12, urokinase plasminogen activator (uPA) receptor (uPAR), and cathepsin V), protein chaperones (heat shock protein (Hsp) 60, Hsp70, and Hsp90), extracellular matrix proteins (collagen, usherin, fibrillins, and laminin), and transcription-regulating proteins (MAPKAPK2, NF-κB, IκB, STAT4, v-Myc, ONECUT 2, and REST) were also down-regulated upon treatment of SB203580. Interestingly, expressions of a few anti-apoptotic proteins (PS-2, caspase 7, and TRABID protein) and anti-growth/mitotic repressor proteins (Spry-4, activin A receptor, SOCS5, wee1, and homolog of checkpoint 1) (Supplemental Tables 7 and 8) were induced, while expression of a few anti-apoptotic proteins (TNF receptor-associated factor 1 and DNA ligase III) (Table IV) were down-regulated upon treatment of SB203580. Over a hundred proteins were listed as miscellaneous proteins. These are proteins that may play roles other than the 12 listed groups including a large number of metabolic enzymes such as NADH2 dehydrogenase and ATPase. Although concordance between microarray and proteomic analysis was low (~20%) (Supplemental Tables 1–8), functional analysis of the differentially expressed genes and proteins showed a similar pattern of changes, especially those involved in growth regulation and gene transcription.

**Identification of Components of Multiple Pathways Involved in Growth/Survival and Invasion**—We focused our attention on protein components of signaling pathways that were differentially expressed in response to SB203580 treatment. Our analysis showed that protein components of multiple pathways regulating a number of cellular activities, including growth, cell cycle progression, and invasion, were modulated by SB203580 treatment (Fig. 6). The identified growth/survival-associated pathways include the p38 MAPK/MAPKAPK2 pathway, p38 MAPK/NF-κB pathway, IL-6/phosphatidylinositol 3-kinase (PI3K) pathway, MKK/JNK pathway, Ras/Raf pathway, and the WNT/Frizzled pathway. Expression of these proteins and several extracellular growth factors/growth-associated proteins (IGF-2, EGF like2, FGF-6, and WNT proteins) were down-regulated upon SB203580 treatment (Table IV). To further confirm this observation, we performed Western blot analysis to determine both kinase-active and/or total protein levels in nine of these pathway-associated proteins at 0 (control), 3, and 21 h of SB203580 treatment (Fig. 7). The expression ratios of these proteins normalized to the loading control between control and treatment (3 or 21 h) were shown in Table V. Expression ratios of total protein levels of all nine proteins (NF-κB, MAPKAPK2, IL-6, IGF2, FGF6, Frizzled2, MKK4, JNK3, and Raf1) determined by Western blots were consistent with those determined by quantitative proteomics. In addition, kinase-active forms of MAPKAPK2, NF-κB, JNK3, and Raf1 were also significantly reduced at both 3 and 21 h of treatment (Fig. 7), indicating that their activities were also inhibited by the p38 MAPK inhibitor SB203580. The kinase-
active form of MKK4 was not significantly reduced by SB203580, suggesting that other isoforms of MKK may be involved in the regulation of JNK3 activity. Perturbation of signaling pathways involving tumor invasion by p38 MAPK inhibition was also demonstrated in our proteomic data. Inhibition of p38 MAPK by SB203580 resulted in down-regulation of five invasion/motility-associated proteins at 3 h and six at 21 h of treatment (Table III), including the down-regulation of uPAR, MMP-12, and cathepsin V (Table IV).

Similarly, microarray analysis of the response to SB203580 treatment also showed disruption of multiple signaling pathways involved in growth/survival and invasion. Several growth factors/cytokines and their receptors were down-regulated at 21 h of SB203580 treatment, including EGF, IL-8, FGF receptor, and the hepatocyte growth factor receptor c-Met (Supplemental Table 2). Several Ras-like genes and NF-κB were also down-regulated at both 3 and 21 h of treatment (Supplemental Tables 1 and 2). Four (3 h) and five (21 h of treatment) invasion or cell motility-related genes, including MMP-2 and RHAMM at both time points (3 and 21 h), were underexpressed after SB203580 treatment (Supplemental Tables 1 and 2). These results suggest the disruption of diverse signal-

### Table IV

| Functional groups | 3 h of treatment | 21 h of treatment |
|-------------------|------------------|------------------|
| Growth-related    | IL-6, FGF6, IGF2, WNT5A/8D Ras, Raf, PI3K, JNK3 | IL-6, EGF-like2, WNT8D/10B Rho 7, Ras activator 3 |
| Anti-apoptosis    | TNF receptor-associated factor 1 | DNA ligase III |
| Biosynthesis      | Ribosomal proteins | Ribosomal proteins |
| Cytoskeleton      | β-Actin | Actin, keratins |
| Tumor invasion/cell mobility | MMP-12 | uPAR, cathepsin V |
| Transcription factors | STAT4, Onecut 2, AT-binding transcription factor 1 | MAPKAPK2, NFκB, IκB, REST, NF-κB |
| Other signaling proteins | Ephrin receptor EphB1 | Ephrin receptor EphB6 |
| Chaperone proteins | Hsp60/70 | Hsp60/70/90 |
| Extracellular matrix | Usherin, Fibrillin 2 | Collagen α, Fibrillin 1 |
| Hypothetical proteins | D2005.5 | KIAA0555 |
| Miscellaneous | NADH2 dehydrogenase | 26S ATPase |

*The short-listed proteins are selected from Supplemental Tables 5 and 6.*

**Fig. 6.** Regulation of multiple pathways by SB203580 via p38 MAPK-dependent and/or -independent mechanisms. All bolded or italic proteins were down-regulated following exposure of transformed FL cells to the p38 MAPK inhibitor SB203580. Bolded letters represent proteins detected at 3 h of SB203580 treatment. Italic letters indicate proteins detected at 21 h of SB203580 treatment. Bolded and italicized letters indicate proteins identified in both experiments.

**Fig. 7.** Western blot analysis of several growth and survival-associated proteins in response to p38 MAPK inhibitor SB203580. The results showed a representative of duplicate experiments. Expression ratio between control and treated of each protein was measured by densitometry, normalized to the loading control, and listed in Table V. Sources of antibodies used are listed in “Experimental Procedures.” Phosphorylated proteins indicate the kinase-active forms of the proteins.
Expression ratios of proteins determined by quantitative proteomics and Western blot analysis

| Common name | Protein ratio | By MS* | By IB* |
|-------------|--------------|--------|--------|
|             | 3 h 21 h     | 3 h 21 h |
| p38MAPK     | Total        | NT A  | NT     |
|             | Kinase active| NT B  | NT     |
| NF-xB       | Total        | –      | –      |
|             | Kinase active| –      | –      |
| MAPKAPK2-2  | Total        | –      | –      |
|             | Kinase active| –      | –      |
| IL-6        | –            | –      | –      |
| IGF-2       | –            | NT     | –      |
| FGF-6       | –            | NT     | –      |
| WNT8d       | –            | –      | NT     |
| MKK4        | Total        | –      | –      |
|             | Kinase active| –      | –      |
| JNK3        | Total        | –      | –      |
|             | Kinase active| –      | –      |
| Raf         | Total        | –      | –      |
|             | Kinase active| –      | –      |
| Frizzled2   | –            | NT     | –      |
| uPAR        | –            | NT     | NT     |
| MMP12       | –            | NT     | NT     |

*Negative value indicates fold decrease of protein levels, while positive value indicates fold increase of protein levels following SB203580 treatment. All ratios represent total levels unless specified as kinase-active form.

Sisc was 0.72, which indicates good reliability of our proteomic data.

**DISCUSSION**

Transformation of FL to aggressive diffuse large B cell lymphoma involves accumulation of molecular abnormalities including t(14;18)(q32;q21) (58, 59), resulting in dysregulated expression of the antiapoptotic BCL-2 (60) and secondary genetic alterations involving p53 (61, 62), p16INK4A (63–65), c-myc (66), and BCL-6 (67). Our previous work demonstrated the utility of cDNA microarray analysis in identifying genes in the Ras/p38MAPK pathway as potential targets for therapy in a subset of transformed FL (1). Our major aim in the current study was to elucidate the cellular consequences of SB203580 exposure in transformed FL. In this regard, we have identified hundreds of differentially expressed genes and proteins in response to a widely used p38 MAPK inhibitor SB203580 (50–54, 68) in a transformed FL-derived cell line (OCI Ly-1) by quantitative proteomics and microarray analysis. We have also shown that SB203580 inhibited the kinase activity of p38 MAPK and JNK, the most common stress-activated protein kinases (69), indicating that our observed transcriptional and translational responses are the results of specific kinase inhibition but not caused by stress-associating signaling. Our results show the widespread effects of SB203580 treatment associated with growth inhibition of transformed FL cells. Furthermore, we demonstrate dysregulation of multiple signaling pathways involved in growth/survival and tumor invasion in transformed FL.

Components of many signaling pathways involving p38 MAPK were identified by our quantitative proteomic analysis and Western blot analysis. Of special interest were the p38 MAPK/MAPKAPK2, p38 MAPK/NF-κB, and p38 MAPK/STAT4 pathways. The p38 MAPK/MAPKAPK2 and p38 MAPK/NF-κB pathways are involved in multiple cellular activities in various cell types, including the regulation of cytokine and growth factor production (46, 70–72), and regulation of invasion-related genes such as uPA (31, 73, 74) and MMPs (75, 76). The p38 MAPK/STAT4 pathway is known to mediate the development of Th1 immune response induced by IL-12 (30). Several other transcription regulators including v-Myc, ONECUT2, and REST were also down-regulated in response to SB203580 treatment. Direct involvement of these proteins in p38 MAPK signaling has not been reported previously; however, the roles of v-myc and ONECUT2 in B cell development and neoplasia have been well documented (77, 78). These proteins may also serve as downstream targets of p38 MAPK in transformed FL cells. In addition, we also observed down-regulation of several growth factors (EGF like2, FGF6, IGF2/IGF2R, and IL-6) and components of growth factor-regulated pathways (IL-6/PI3K, Ras/Raf, MKK/JNK, and WNT/Frizzled) by SB203580. The regulation of IL-6 expression (45, 47, 79) and PI3K activation (80) by p38 MAPK has been demonstrated previously. Regulation of IL-6 production (45, 79) or IL-6 mRNA stability (47) by neuropeptide substance P and bradykinin or IL-4 is p38 MAPK dependent. Activation of the PI3K/Akt pathway by TGF-β is mediated by p38 MAPK, possibly through p38 MAPK-dependent induction of an autocrine growth factor (80). It is conceivable that the production of EGF like2, FGF6, IGF2, and WNT family proteins in the transformed FL cells are indirectly controlled by p38 MAPK.
activity through the effect of its downstream effectors (such as NF-κB, MAPKAPK2, or STAT4), similar to the regulation of IL-6 (46), IL-8 (46, 70, 71), and TNF-α expression (72). These growth factors and cytokines may subsequently exert their effects on growth and survival via activation of the PI3K/protein kinase B (PKB), Ras/Raf, MKK/JNK, and β-catenin/TCF pathways (Fig. 6). Regulation of MKK/JNK and WNT/FRizzled by p38 MAPK has not been demonstrated previously; however, these proteins are frequently associated with cell growth and survival (81–83). Based on the fact that p38 MAPK often exerts its effect through transcription regulators, and acts downstream of Ras and MKK signaling pathways (28, 29, 84, 85), we hypothesize that the dysregulation of the diverse overlapping signaling pathways by the p38 MAPK inhibitor SB203580 is mediated by its downstream effectors (MAPKAPK2, NF-κB, STAT4, v-myc, and ONECUT2) regulating gene expression including the expression of growth factors, cytokines, and invasion-associated genes (Fig. 6). Interestingly, our microarray data also showed down-regulation in the expression of IL-8, TGF-β, EGF, FGF receptor, and NF-κB (Supplemental Tables 1 and 2). This suggests that regulation of gene expression by p38 MAPK may occur at the transcriptional and/or post-transcriptional level, as suggested by a number of previous studies (35, 47, 86–92). Our data show that many p38 MAPK-mediated signaling pathways that have been previously identified are dysregulated in transformed FL cells. In addition, our data suggest the involvement of other genes and proteins that may represent novel mediators of the p38 MAPK pathway that are relevant in B cell lymphomagenesis and transformation.

Many of the growth factor-mediated signaling pathways identified by our study have been previously shown to play a role in the pathogenesis of lymphomas. IL-6 is a requisite and cooperative growth factor of non-Hodgkin’s lymphoma cells (93–95). Elevated transcript levels of PI3K and three Ras family genes are found in transformed FL but not FL (1). In addition, p110α, the catalytic subunit of PI3K, serves as an important mediator for antigen-receptor signaling in the activation and development of both T and B cells (96). Ectopic overexpression of active Ras induces the metastatic and invasive potential of a T lymphoma cell line (97) and also plays a role in the development of B cell lymphoma (98). The protooncogene Raf, a downstream effector of Ras, is also involved in the development of lymphoma and leukemia (99). The role of deregulated WNT signaling in the development of B and T cell neoplasia has also been documented (100, 101). Treatment of WNT3a in multiple myeloma cells up-regulates β-catenin and induces striking morphological changes that are associated with increased cell motility and metastatic potential (100). Expression of transcription factor TCF-1, a downstream effector of the WNT/β-catenin pathway, is detected in the vast majority of peripheral T cell lymphoma (101). In B cell chronic lymphocytic and myeloid leukemia, elevated level of FGF2 is observed and may modulate cell growth through the activation of the MEK/ERK pathway (102). Furthermore, translocation of FGF3/4 has also been reported in B cell lymphoma (103). The role of EGF receptor in the transformation of a mouse pre-B cell lymphoma has also been reported (104).

Our data identified several growth factors and related signaling pathways such as IGF and MKK/JNK pathways that have not been previously associated with development of lymphomas. However, the role of the IGF/Ras/JNK pathway in the regulation of growth and survival in neuroblastoma cells (105) and cervical cancer cells (81) has been reported. Further functional studies of these proteins in the biology of transformed FL cells will provide more insights regarding their pathogenetic role in FL transformation.

p38 MAPK also plays an important role in tumor invasion and angiogenesis by regulating the expression or activity of uPA (31, 73, 74) and MMPs (75, 76). The p38 MAPK/MAPKAPK2 pathway regulates mRNA stability of uPA in invasive breast cancer cells (31, 74). In our studies, we showed down-regulation of MMP-12 (protein, Supplemental Table 5), MMP-2 (mRNA, Supplemental Tables 1 and 2), and uPAR (protein, Supplemental Table 6) by the p38 MAPK inhibitor SB203580. These genes have all been associated with invasion and/or angiogenesis of several tumors (106–108). Our data suggest a role for p38 MAPK in the invasive properties of transformed FL cells.

In contrast to the majority of proteins that were down-regulated, SB203580 administration resulted in up-regulation in expression of several groups of proteins (Fig. 8) (Supplemental Tables 7 and 8), including the suppressor of cytokine signaling 5 (SOCS5), mitotic inhibitors (wee1 tyrosine kinase and homolog of checkpoint 1), growth repressors (Spry-4, NF1, and WD repeat-containing protein), repressor of tumor metastasis (TIMP2), and the c-cbl ubiquitin ligase. Because these proteins are associated with growth inhibition, cell cycle arrest, or tumor repression (109–117), our data suggest a possible role for p38 MAPK-mediated repression of anti-growth, anti-mitosis, and anti-metastasis proteins in the progression of B cell lymphoma.

Several cellular effects have been attributed to the p38 MAPK-independent actions of SB203580. For instance, SB203580 has been shown to exhibit inhibitory activity against PKB via direct inhibition of phosphoinositide-dependent kinase 1 (PKD1) but not via inhibition of PI3K at moderately high drug concentrations (>1–3 μM) (118). Similarly, SB203580 has been shown to affect the activity of the hepatic cytochrome P450 enzymes (119). This activity is explained by the fact that pyridine and imidazole components of the SB203580 compound are ligands for the heme iron of cytochrome P450 (120). Interestingly, our studies revealed 5.2-fold induction of an isofrom of cytochrome P450, supporting the previously described interaction between SB203580 and cytochrome P450. Several metabolism-associated cellular responses to SB203580 treatment were also demonstrated by
Proteomic and Transcriptional Consequences Associated with SB203580 Treatment

Fig. 8. Induction of several protein groups by p38 MAPK inhibitor SB203580. All listed proteins were up-regulated following exposure of transformed FL cells to the p38 MAPK inhibitor SB203580. Bolded letters represent proteins detected at 3 h of SB203580 treatment. Italic letters indicate proteins detected at 21 h of SB203580 treatment.

In our studies. These include the regulation of many energy metabolic proteins (NADH dehydrogenase, ATPases, and ATP transporters) and carbohydrate/amino acid metabolic proteins (aspartate hydrolase, aldehyde reductase, glycerol dehydrogenase, and dihydrolipoamide dehydrogenase) (Supplemental Tables 5–8) with diverse consequences. For instance, NADH dehydrogenase was up-regulated (Supplemental Table 8), while a few ATPases were down-regulated (Supplemental Table 6). The link between metabolic pathways and lymphocyte function has been documented previously (121, 122).

Using an immobilized analogue of SB203580 as a bait in an affinity pull-down approach, Godl et al. recently demonstrated a physical interaction between the pyridinyl imidazole analogue with a complex containing several proteins including some well-established signaling molecules such as JNK1, several heat shock proteins, and metabolic enzymes (123). In this study, we independently observed SB203580-induced differential expression of a number of the same or related proteins including HSP70.1, HSP90, PKNβ, pyruvate kinase, and peroxisomal 3,2-trans-enoyl-CoA isomerase. No differential expression of RICK, GSK-3β, CK1, JNK1, JNK2, and GAK was detected by our proteomic analysis. However, the inhibited kinase activity of JNK3 observed in our studies may also be a direct inhibitory effect of SB203580, because the structure of JNK3 is highly homologous to JNK1 and JNK2, whose in vitro kinase activities are inhibited by SB203580 (123). Differences in cell-type specific expression of these proteins may also explain some of the discrepant results. On the whole, these studies independently and complementarily demonstrate that the widespread cellular effects of SB203580 may be affected through p38 MAPK-dependent and -independent mechanisms.

In summary, our studies demonstrated a global spectrum of transcriptional proteomic changes associated with exposure of a transformed FL-derived cell line OCI Ly-1 to the p38 MAPK inhibitor SB203580, thereby supporting the existence of numerous deregulated signaling pathways in the pathogenesis of transformed FL. While direct comparison of the changes of specific transcripts could not be performed with their corresponding proteins because our cDNA array contained only 9,600 clones (a substantial number of which are uncharacterized ESTs), the trends of differential expression were maintained across the broad transcriptional and proteomic datasets reflecting functional groups and pathways affected by SB203580 treatment in the lymphoma cells. Indeed, profound biological activities were altered upon SB203580 treatment, as demonstrated by our analysis of the differentially regulated functional groups. Analysis of functional groups of differentially expressed proteins and Western blot analysis of selected proteins also identified several known and novel p38 MAPK-mediated signaling pathways involved in cell growth, survival, transformation, and invasion. Additionally, our data also implicate the involvement of several SB203580-targeted p38-independent pathways in the pathogenesis of FL transformation. These data demonstrated a potential role of p38 MAPK deregulation in B cell lymphomagenesis and progression. Our studies demonstrate the utility of functional genomic approaches in the comprehensive identification of genes/proteins and signaling pathways that are involved in disease pathogenesis, and the potential for exploitation of the new knowledge thereby obtained in approaches to the specifically targeting deranged signaling pathways.

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Proteomic and Transcriptional Consequences Associated with SB203580 Treatment
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