Kinome Profiling for Studying Lipopolysaccharide Signal Transduction in Human Peripheral Blood Mononuclear Cells

The DNA array technique allows comprehensive analysis of the genome and transcriptome, but the high throughput array-based assessment of intracellular signal transduction remains troublesome. The goal of this study was to test a new peptide array technology for studying the activity of all kinases of whole cell lysates, the kinome. Cell lysates from human peripheral blood mononuclear cells before and after stimulation with lipopolysaccharide were used for in vitro phosphorylation with [γ-33P]ATP arrays consisting of 192 peptides (substrates for kinases) spotted on glass. The usefulness of peptide arrays for studying signal transduction was demonstrated by the generation of the first comprehensive description of the temporal kinetics of phosphorylation events induced by lipopolysaccharide stimulation. Furthermore, analysis of the signals obtained suggested activation of p21Ras by lipopolysaccharide, and this was confirmed by direct measurement of p21Ras GTP levels in lipopolysaccharide-stimulated human peripheral blood mononuclear cells, which represents the first direct demonstration of p21Ras activation by stimulation of a Toll receptor family member. Further confidence in the usefulness of peptide array technology for studying signal transduction came from Western blot analysis of lipopolysaccharide-stimulated cells, which corroborated the signals obtained using peptide arrays as well as from the demonstration that kinase inhibitors affected peptide array phosphorylation patterns consistent with the expected action of these inhibitors. We conclude that this first metabolic array is a useful method to determine the enzymatic activities of a large group of kinases, offering high throughput analysis of kinase activity. Hence, in principle an array exhibiting specific kinase activity may be regarded as the minimal transcriptome. Only a small portion of the transcripts present in the cell determines the identity of the cell, and these critical transcripts are expressed at low levels. Therefore, small changes in the expression profiles in the transcriptome can lead to large changes in enzymatic profile of the cell leading to significant differences in cell functioning. Thus, a comprehensive description of cellular metabolism may be more useful than such a description of the genome and transcriptome.

Array technology has not yet been adapted to measure enzymatic activity in whole cell lysates, but progress has been made with the preparation of protein chips for the assessment of protein substrate interactions and the generation of peptide chips for the appraisal of ligand-receptor interactions and enzymatic activities. Recently, Houseman and Mrksich (14) showed that employing peptide chips, prepared by the Diels-Alder-mediated immobilization of one kinase substrate (for the non-receptor tyrosine kinase c-Src) on a monolayer of alkanethiols on gold, allows quantitative evaluation of kinase activity. Hence, in principle an array exhibiting specific consensus sequences for protein kinases across the entire kinome (the combined activity of all cellular kinases) would allow a more comprehensive detection of signal transduction events in whole cell lysates. Obviously, employing this kind of array technology for this purpose would allow faster and more inclusive analysis of cellular metabolism in comparison to currently available technology, which focuses on the static determination of the relative concentration of metabolites but does not address the actual activity of various cellular signaling pathways.

The above-mentioned considerations prompted us to test the usefulness of peptide arrays containing spatially addressed mammalian kinase substrates for studying the kinome in a cellular context. We show that such peptide arrays allow us to make a comprehensive description of the phosphorylation events induced by lipopolysaccharide (LPS) stimulation of peripheral blood mononuclear cells. Furthermore, analysis of the results revealed a role of p21Ras in LPS signal transduction, and this finding was confirmed by a pull-down assay. Thus the peptide array technology enabled us to identify the first example of p21Ras activation by a member of the toll receptor family.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—The catalytic subunit of protein kinase A was purchased from Promega (V5161).

**Single Kinase Analysis on Peptide Array**—50 μl of the protein kinase

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* The abbreviations used are: LPS, lipopolysaccharide; PKA, protein kinase A; PBMC, peripheral blood mononuclear cell; IMDM, Iscove's modified Dulbecco's medium; MAP, mitogen-activated protein.
A (PKA) incubation mix (500 ng/ml PKA catalytic subunit, 50 mM HEPES, pH 7.4, 20 mM MgCl₂, 10% glycerol, [\gamma^{33}P]ATP, 0.01% (v/v) Brij-35, 0.01 mg/ml bovine serum albumin, [\gamma^{33}P]ATP (1000 kBq)) was added to the glass slide and incubated at 30 °C for 90 min in a humidified oven. After incubation the glass slide is washed twice in 2M NaCl, twice in demineralized H₂O, and air-dried.

Peripheral Blood Mononuclear Cells Isolation—Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of healthy volunteers using standard density gradient centrifugation over Ficoll-Paque Plus (Amersham Biosciences AB), followed by washing and resuspension in IMDM supplemented with 10% fetal bovine serum, penicillin, streptomycin, and amphotericin (hereafter referred to as complete IMDM).

Western Blot Analysis—For Western blot samples, 10⁶ PBMCs were suspended in 1 ml of complete IMDM, and LPS stimulations were 15-min incubations (37 °C, 5% CO₂) with 100 ng/ml LPS, Escherichia coli, serotype 0111:B4. Stimulations were terminated by an ice-cold phosphate-buffered saline wash. Cells were subsequently pelleted, lysed, denatured (5 min at 95 °C), and stored at −20 °C. 10⁶ PBMCs lysed in 200 μl of SDS sample buffer (62.5 mM Tris-HCl (pH 6.8 at
25 °C), 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% (w/v) bromophenol blue, 25 μl of which was separated on 12% SDS-PAGE gels and transferred to nitrocellulose membranes. Phosphospecific antibodies for immunoblotting were from Cell Signaling and included phospho-p38 MAP kinase (catalog number 9211), phospho-p44/42 MAP kinase (catalog number 9101), phospho-PKC/α (catalog number 9378), phospho-MEK1/2 (catalog number 9121), phospho-Raf (catalog number 9424), phospho-Akt (catalog number 9271), and phospho-SAPK/JNK (catalog number 9252). Non-phosphorylated Ras-activation was chased from Santa Cruz Biotechnology (Santa Cruz, CA). All antibodies were used in accordance with the supplier’s protocol, and images were revealed with a Lumi-Imager (Roche Applied Science) using the chemiluminescence substrate Lumilight (Roche Applied Science).

Peptide Array Analysis—For kinase array samples, 10^7 PBMCs were suspended in 5 ml of complete IMDM. Stimulations were terminated by an ice-cold phosphate-buffered saline wash. PBMCs were lysed in 200 ml of cell lysis buffer (20 mM Tris- HCl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM MgCl2, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM NaF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF) and the volume of the cell lysate was equalized with distilled H2O. The cell lysates were subsequently cleared on a 0.22-m filter. Peptide array incubation mix was produced by adding 10 μl of filter-cleared activation mix (50% glycerol, 50 μM [γ-32P]ATP, 0.05% w/v Brij-35, 0.25 mM Na3VO4, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM NaF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF) and bovine serum albumin, [γ-32P]ATP (1000 KBBq)). Next, the peptide array mix was added onto the chip, and the chip was kept at 37 °C in a humidified stove for 90 min. Subsequently the peptide array was washed twice with Tris-buffered saline with Tween, twice in 2 M NaCl, and twice in demineralized H2O and then air-dried. The experiments were performed three times in duplicate.

Analysis of Peptide Array—The chips were exposed to a phosphorimager plate for 72 h, and the density of the spots was measured and analyzed with array software.

Ras Activation Assay—The Ras Activation Assay kit (Upstate Biotechnology, Lake Placid, NY) was used following the supplier’s instructions. Determination of protein concentration required by the Ras Activation Assay kit was made with the BCA Protein Assay Kit (Pierce) according to the supplier’s instructions.

RESULTS

Peptide Array Design and Construction—In silico analysis of the Phosphobase resource enabled identification of consensus amino acid phosphorylation sequences for most kinases present in mammalian genome (15, 16). Further analysis of this set of kinase substrates revealed that the mean amino acid residue length that achieves an optimal specificity/sensitivity ratio for substrate phosphorylation was nine amino acids, and thus for building an array nonapeptides were employed. Arrays were constructed by chemically synthesizing soluble pseudo-peptides, which were covalently coupled to glass substrates (extensively described in the supplemental data). This array consisted of 192 peptides (denominated A1–P12), providing kinase substrate consensus sequences across the mammalian kinome.

TABLE II

| Kinase Substrate | Control | LPS |
|-----------------|---------|-----|
| AUTO PKG       | 2500    | 8100 |
| JAK STAT1a/b  | 248     | 704  |
| c-Src PTP-2C   | 287     | 5900 |
| PKA/Ca2+ Trp-S-MO | 1164  | 23925 |
| Cytokine receptor FFS | 270     | 5512 |
| PKA Glycogen synthase | 795 | 7980 |
| PKC alF4E     | 4494    | 41272 |
| PKA v-Rel     | 291     | 2574 |
| PKA P450      | 2412    | 19980 |
| CKII LamBR    | 214     | 1736 |
| Cdc2 CK II    | 268     | 1728 |
| PKA MBP       | 280     | 1250 |
| CKII TOPII    | 201     | 900  |
| PKC ACC-a     | 2760    | 10680 |
| PKA HSP27     | 288     | 1072 |
| CKII Lamin D  | 242     | 822  |
| CK1 Glycogen synthase | 5166 | 13386 |
| p38 MAPKAP2   | 10,168  | 21,440 |
| Src Annexin-2 | 4438    | 8745 |
| p34cdc2 CK II | 320     | 580  |
| Glycogen synthase | 9758 | 17052 |
| PKC BICKS     | 23,400  | 38,088 |
| PKA MBP       | 536     | 822  |
| v-Fps Caldesmon | 16,002 | 22,800 |
| MBP           | 26,208  | 38,442 |
| PKC c-RAF     | 10,764  | 13,840 |
| PKG           | 108     | 230  |
| eIF4F         | 41,234  | 41,272 |
| p34cdc2 AP-1  | 460     | 548  |
| Vimentin      | 26,240  | 30,888 |
| Glycogen synthase | 16,920 | 19,500 |
| Cdc2 Vimentin | 192     | 214  |
| Myosin light chain-2 | 48,400 | 53,482 |
| p34cdc2 c-Src | 230     | 248  |
| MBP           | 60,630  | 62,900 |
| GSK3          | 312     | 320  |
| CK II TOPII   | 274     | 280  |
| PKA Tau       | 340     | 340  |
To allow assessment of possible intra-experimental variability in substrate phosphorylation, on each separate carrier the array was spotted two times using a biorobotics microgrid spotter equipped with 100 micron split pins. Peptides were spotted onto and subsequently covalently coupled to branched hydrogel polymer-coated glass slides (supplemental data). Spotted slides were stored at 4 °C. The final physical dimensions of the array were $19.5 \times 19.5$ mm with each peptide spot having a diameter of $350 \mu m$ and peptide spots being 750 $\mu m$ apart.

**Single Kinase Analysis**—If the design of our peptide array was appropriate, addition of a purified kinase in the presence of ATP should result in the phosphorylation of the appropriate consensus peptide sequences without concomitant phosphorylation of other sites (see Table I). To allow assessment of possible intra-experimental variability in substrate phosphorylation, on each separate carrier the array was spotted two times using a biorobotics microgrid spotter equipped with 100 micron split pins. Peptides were spotted onto and subsequently covalently coupled to branched hydrogel polymer-coated glass slides (supplemental data). Spotted slides were stored at 4 °C. The final physical dimensions of the array were $19.5 \times 19.5$ mm with each peptide spot having a diameter of $350 \mu m$ and peptide spots being 750 $\mu m$ apart.

**FIG. 3.** Temporal phosphorylation of different kinase substrates in PBMCs stimulated with LPS. Peripheral blood mononuclear cells were stimulated with LPS (100 ng/ml), and lysates were obtained from the cells after 0, 5, 15, 30, and 60 min of LPS stimulation (as described under “Experimental Procedures”). The arrays were quantified, the values of unstimulated cells were set at 1, and the other time points were compared with the unstimulated values. The graphs show the phosphorylation profiles of 20 different substrates with their respective S.E. after 0, 5, 15, 30, and 60 min of LPS stimulation. The graphs A–P are substrates that have been reported to be involved in LPS/tumor necrosis factor signaling or in MAP kinase signaling, and the graphs Q–T are of substrates that are not commonly associated with LPS signal transduction.
metabolic arrays reveal kinetics of LPS signal transduction

- LPS + LPS

A Comprehensive Description of LPS-induced Phosphorylation Events—The possibility to study a wide range of kinases in parallel makes it possible to make a comprehensive description of the temporal characteristics of LPS-induced phosphorylation, revealing the sequential activation and deactivation of the various kinases. Hence we stimulated human PBMCs for 5 min, 15 min, 30 min, and 60 min and analyzed the effects on kinase activity employing peptide arraying, and the results show different kinetics for phosphorylation of a variety of substrates (Fig. 4). Confirming the results described above, especially enhanced phosphorylation was detected for substrates of various MAP kinases, which are known to be involved in LPS-induced p44/p42 MAPK (18–20) (e.g. Raf, myelin basic protein). A peptide containing the STAT-1α/β phosphorylation site incorporated more radioactivity when incubated with lysates from LPS-stimulated cells (Fig. 4) because STAT-1α/β phosphorylation is a known cellular effect of LPS (21–24). Also, peptides derived from NFB proteins were also phosphorylated (Fig. 4). Interestingly, the phosphorylation of these peptides peaked in 5–15 min and came back to basal levels after 60 min of LPS stimulation, which is in agreement with the expected time course. Remarkably, Bruton’s tyrosine kinase, a member of the Tec kinase family, was recently reported to be involved in LPS signaling (25), and we found corresponding phosphorylation of peptide corresponding to its activation site. In addition, phosphorylation of peptides derived from cytoskeletal proteins became even more pronounced as in unstimulated cells (vimentin, lamin D, and lamin B1), in agreement with the effects of LPS on cell morphology and endocytosis (Fig. 4) (19, 26). The induction of eIF-4F corresponds well to the induction of gene expression by LPS (Fig. 4). Other remarkable effects are the changes in phosphorylation of substrates derived from p450 (CYPII) and α-crystallin (Fig. 4), and results correspond well to data published earlier (27, 28). Interestingly, several peptides derived from proteins that had not been linked to LPS signal transduction as yet also display marked changes in phosphorylation; e.g. Muscarinic Receptor M2, Rhodopsin, NEU (erbB2), and Phox47. However, phosphorylation of these proteins by MAP kinases has been reported, and thus these effects may well be indirect (Fig. 4) (29–32). A picture emerges in which phosphorylation of various substrates is dynamically regulated as a consequence of the LPS stimulation.

Effects of Kinase Inhibitors on Lysates-induced Peptide Array Phosphorylation—To determine whether the glass slide-based

FIG. 4. Western blot controls for LPS-induced changes in peptide phosphorylation. The lysates for SDS-PAGE of peripheral blood mononuclear cells stimulated for 15 min with LPS or untreated cells were obtained in parallel with those used for peptide array phosphorylation, were investigated using a standard Western blotting protocol, and were probed with activation state-specific antibodies according to the supplier’s protocol. The apparent weight of the proteins under our conditions is indicated in the figure.
peptide arrays gave functional and realistic results with respect to phosphorylation, we used the MAP kinase inhibitors PD 98059 and SB 203580. Indeed these inhibitors prevented the phosphorylation of MAP kinase regulated substrates (Fig. 5) (myelin basic protein, MAPKAPK2, c-Jun), and the substrates that were regulated by MAP kinases (muscarinic receptor M2, rhodopsin, NEU (erbB2), and Phox47) were also inhibited (Fig. 5). However other substrates that are not directly

**FIG. 5.** Effects of two MAP kinase inhibitors in the phosphorylation of different kinase substrates in cells stimulated with LPS. Cells were pre-incubated for 1 h with the inhibitors PD (PD98059, 50 \( \mu \)M) or SB (SB203580, 10 \( \mu \)M) and subsequently stimulated with 100 ng/ml LPS; after 0 and 60 min the cells were lysed and analyzed using the peptide array analysis protocol. The arrays were quantified, the values of unstimulated cells were set at 1, and the other conditions were compared with the unstimulated values. The graphs show the phosphorylation level of 20 different substrates with their respective S.E. The first two bars depict normal peripheral blood mononuclear cells, the second two bars are from cells pretreated with PD98059, and the last two bars depict lysates that are pretreated with SB203580. The first bar represents unstimulated cell lysates, and the second is from lysates that are stimulated with LPS. The graphs A–P are substrates that have been reported to be involved in LPS/tumor necrosis factor signaling or in MAP kinase signaling, and the graphs Q–T are of substrates that are not commonly associated with LPS signal transduction.
phosphorylated by MAP kinases are also influenced by the inhibitors, indicating that cross-talk and/or feedback loops between different classes of kinases is possible (STAT-1/α-crystallin, Tec).

Analysis of the Peptide Array Results Reveals a Role for p21Ras Activation in LPS Signal Transduction—Among the most important questions in LPS signal transduction is the molecular mechanism leading to the activation of the Raf/MEK/p42/p44 MAP kinase-signaling cascade. Classically, activation of this cascade is brought about either via the p21Ras route (which is archetypical for receptor tyrosine kinase-coupled receptors) in conjunction with protein kinase C or via the sequential activation of phospholipase C and protein kinase C (which are archetypical for G protein-coupled receptors). Because LPS signals via the Toll-like receptor 4 (20), neither of both possibilities is immediately obvious. However, p21Ras has been reported to be linked to Toll/interleukin-1 receptor domain-dependent signaling indicating that Ras might be activated upon stimulation with LPS (33). Apart from activating the p42/p44 MAP kinase signaling cascade, Ras activation also leads to stimulation of the phosphatidylinositol 3-kinase/protein kinase B pathway as well as to extensive cytoskeletal remodeling. We noticed that in our results LPS induced phosphorylation of peptides that are in accordance with activation of p21Ras as judged from the increase in phosphorylation of peptides associated with the MAP kinase pathway, the phosphatidylinositol 3-kinase pathway, and cytoskeletal proteins. Also other substrates consistent with the activation of p21Ras (MARCKS, Na+/K+ ATPase, Annexin-2; Fig. 6) were phosphorylated after LPS stimulation. This prompted us to look at whether p21Ras might be involved in LPS signaling. Indeed in a p21Ras activation assay we detected increased GTP-bound p21Ras. This marks the first direct identification of p21Ras activation via the stimulation of a member of the Toll-like receptor family. These data show that it is possible to use peptide array technology to characterize changes in cellular metabolism and signal transduction even in a temporal manner and find novel interactions between signaling cascades.

DISCUSSION

We interpret our kinome analysis as a useful and valuable method to determine the enzymatic activities of a large group of kinases. Therefore, kinome profiling could be a realistic possibility and especially interesting as the current metabolomics effort has been hampered by the lack of techniques that allow high-throughput analysis of the flow of cellular metabolism. Current mass spectrometry techniques concentrate on the static determination of metabolite levels rather as the enzymatic activity of the biochemical process leading to these levels. In the present study we focused on kinome profiling, but one can easily imagine arrays for assaying cellular activity with respect to dephosphorylation, acylation, acetylation, ubiquitination (ubiquitome), etc. Thus arraying for enzymatic activities may provide metabolomics with the equivalent of the DNA array analysis for genomics with respect to the possibility to quickly obtain a comprehensive description of cellular metabolism and cellular transcriptome respectively.

At present kinome arraying still suffers from “teething problems.” One of the aspects that may have to be determined is the fact that phosphatases present in the lysate could influence the
amount of phosphorylation; to prevent this we employed a full spectrum of inhibitors. Obviously, however, the net amount of phosphorylation in the cell critically depends on the net activity of kinases and phosphatases, and thus the results obtained may not reflect the actual phosphorylation status of substrates in the cell but rather the amount of enzymatic phosphorylating activity. In essence kinase arraying measures flow and not absolute levels of substrate phosphorylation. In principle, using pre-phosphorylated arrays, it should be possible to measure phosphatase activity as well. It also may be possible to perform experiments without phosphatase inhibitors, but until it is shown that under the in vitro conditions of array phosphorylation kinase and phosphatase activity show similar temporal characteristics, results should be interpreted with caution.

Another possible concern is that less abundant kinases may be more difficult to visualize because more abundant kinase will produce stronger signals but may not be less important for the control of physiological processes. One possible way to alleviate this problem is by using relative activities for each spot. In this manner each spot has its own range, and the differences in intensity are bypassed. In this manner one looks at the fold induction instead of the amount of phosphorylation. The fact that phosphorylation can be circumvented by employing relative activation levels. This may be alleviated by developing specific pseudo-substrates, harboring only one phosphorylation site, and by developing peptides with increased specificity for one kinase. The problem that MAP kinases or other kinases perform a double phosphorylation in the cell but rather the amount of enzymatic phosphorylating activity is by using relative activation levels. In this manner one looks at the fold induction instead of the amount of phosphorylation. The fact that phosphorylation can be circumvented by employing relative activation levels. This may be alleviated by developing specific pseudo-substrates, harboring only one phosphorylation site, and by developing peptides with increased specificity for one kinase.

Further development of this technique is now critically dependent on the generation of peptides having improved specificity for further cellular kinases as well as expansion of the array to include the entire kinome. In particular, a pressing issue is the concern that some peptides can be phosphorylated on more than one spot and that this results in over-phosphorylation of the peptide and therefore overrating the kinase activity. This may be alleviated by developing specific pseudopeptides, harboring only one phosphorylation site, and by developing peptides with increased specificity for one kinase. The problem that MAP kinases or other kinases perform a double phosphorylation can be circumvented by employing relative values in the analysis or other post hoc corrections in the analysis software. It is important to realize that no data are currently available that suggest that peptides with multiple phosphorylation sites for kinases are also phosphorylated on those two sites simultaneously; however the extent of the problem is unknown, and more research may be needed in this direction.

Disregarding these limitations, however, our present study has shown that the kinome reacts dynamically to stimulation with LPS and has helped in identifying p21Ras as a novel signal transducer in LPS signaling. Thus we feel that peptide arraying for kinome-wide analysis of biologically relevant samples is a highly promising tool for studying the biochemical changes underlying cellular signal transduction.

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