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Published in:
Journal of Biological Chemistry

Publication status and date:
Published: 19/11/2004

DOI (link to publisher):
10.1074/jbc.M405028200

Document Version
Publisher's PDF, also known as Version of record

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Citation for the published version (APA):
Diks, S. H., Kok, K., O'Toole, T., Hommes, D. W., Van Dijkenii, P., Joore, J., & Peppelenbosch, M. P. (2004). Kinome profiling for studying lipopolysaccharide signal transduction in human peripheral blood mononuclear cells. *Journal of Biological Chemistry*, *279*(47), 49206-49213. https://doi.org/10.1074/jbc.M405028200

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 47, Issue of November 19, pp. 49206–49213, 2004
Printed in U.S.A.
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Kinome Profiling for Studying Lipopolysaccharide Signal Transduction in Human Peripheral Blood Mononuclear Cells* Ⓢ

Received for publication, May 5, 2004, and in revised form, September 7, 2004
Published, JBC Papers in Press, September 7, 2004, DOI 10.1074/jbc.M405028200

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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-^{33}P]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 effected 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 cellular metabolism and signal transduction.

Massive parallel analysis using array technology has become the mainstay for the analysis of genomes and transcriptomes (1–5). Since the determination of the transcriptome, the understanding of cellular functioning has improved dramatically. Novel insights have led to the notion that the majority of the transcriptome is necessary to keep a cell functioning and could 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 (6). 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 (7–10) and the generation of peptide chips for the appraisal of ligand-receptor interactions and enzymatic activities (11–13). 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 alkanethiolates 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)[1] 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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[1] 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.

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TABLE I

Sequences of substrate peptides and their location on the peptide array

All sequences are derived from the Phosphobase resource (phospho.elm.eu.org). The mean amino acid residue length that achieves an optimal specificity/sensitivity ratio for substrate phosphorylation is nine amino acids. The array consists of 192 peptides, denominated A1-P12, of which 8 are controls (rhodamine-labeled irrelevant peptides for quality control of the spotting process). On each carrier these 192 peptides were spotted twice.

	A	B	C	D	E	F	G	H
1	Control	KRPSERAKA	LQDDYEDMM	PLSRTLSVS	Control	KRPSDRAKA	LQAISPKQS	PLAGSPVIA
2	PVSPSLVQG	KKPPTPPPE	KTETSQVAP	LSGFSFKKN	PVPKSPVEE	KKLGSKKPQ	KSRWSGSQQ	LSGESDLEI
3	PQPEYVNQP	Control	KRKVSSAEG	LNDSSEEED	PPSPSLSRH	Control	KRKRSRKES	LMDKYHVDN
4	PAAVSEHGD	PTKRSPTKR	KKIDSFASN	KSLNYIDLD	LYSSSPGGA	PTKRSPQKG	KKDVTPVKA	KSKISASRK
5	LRGRSFMNN	PPRRSSIRN	LRRASLRG	KRFGSKAHM	LRGPSWDPF	PPEKTEEEE	LGSPLRRR	KREASLDNQ
6	KVPQTPLHT	LSRFSWGAE	PSRRSRSRS	KKAESPVKE	KTTASTRKV	LSLDSQGRN	PSQRSKYLA	KISITSRKA
7	PRTPGGRR	KQPIYIVME	LGGGTFDIS	PEGDYEEVL	KRKQISVR	KPGFSPQPS	LFRLSEHSS	PASQTPNKT
8	PSPKTPPGS	KGTGYIKTE	KRRNSEFEI	LRRASLGAF	PSLPTPPTR	KGQESFKKQ	KRRLSFSET	LRRASLGAA
9	PKEVYDVML	QGTLSKIFK	KNDKSKTWQ	LDDQYTSSS	PKDPSQRRR	QEQEYVQAV	KMKDTDSEE	LASSSKEEN
10	LSDDSFIED	PRRNSRASL	KGATSDEED	KRQSSTSNA	LRSPSWEPF	PRRDSTEGF	KEDTYTAHA	KRPTQRAKY
11	LLQDSVDFS	PGGSTPVSS	QEGDTDAGL	KLINSIADT	LLPMSPEEF	PFKLSGLSF	QDPVSPSLV	KKSWSRWTL
12	KRSLSEMEI	LRRPSDQAV	PRKGSPRKG	KAQEYFNIK	KRSGSVYEP	LRRLSTKYR	PRKGSPKRG	KAKVTGRWK

	I	J	K	L	M	N	O	P
1	Control	KRPSARAKA	LPVPSTHIG	PKRGSGKDG	Control	KRNSSPPPS	LNRMSFASN	PKKGSKKAV
2	PVPEYINQS	KKKGSGEDD	KSRPSLPLP	LSELSRRRI	PTRHSRVAE	KKKFSFKKP	KSPAKTPVK	LSEHSSPEE
3	PPSAYGSVK	Control	KRKQISVRG	LMAPSEEDH	PPSAYATVK	Control	KRKNSILNP	LLRPSRRVR
4	LVVASAGPT	PTGTTPQRK	KKDASDDLD	KSFGSPNRI	LSRHSSPHQ	PSSTSSSSI	KKASFKAKK	KRSNSVDTS
5	LRAPSWIDT	PMRRSVSEA	LGSALRRR	KRAASPRKS	LQRYSSDPT	PLTPSGEAP	LELSDDDD	KQSPSSSPT
6	KTSPSSSPA	LSGLSFKRN	PSPSSRVTV	KISASRKLQ	KTRSSRAGL	LSGFSFKKS	PSPKYPGPQ	KIQASFRGH
7	KQISVRGL	KNIVTPRTP	LEKKYVRRD	PASPSPQRQ	KQGSGRGL	KNDYYRKRG	LDPLSEPED	PASAYGSVK
8	PSEKSEEIT	KGHEYTNIK	KRRGSVPIL	LRMFSFKAP	PSAPSPQPK	KGGSYSQAA	KRRDYLDLA	LRKVSKQEE
9	PINGSPRTP	QEPGSGPPE	KLSPSPSSR	KYRKSSLKS	PGPQSPGSP	QEGLYNELQ	KLRRSSSVG	KYLASASTM
10	LRRSSSVGY	PRPASVPPS	KDIGSESTE	KRPSGRAKA	LRRPSDQEV	PRMPSLSVP	KASASPRRK	KRPSFRAKA
11	LKLASPELE	PETVYEVAG	PWRITDNEL	KKRLSVERI	LIEDAEYTA	PENDYEDVE	PWQVSLRTR	KKRFSFKKS
12	KRRSSSYHV	LRRFSLATM	PRHLSNVSS	KAKTTKKRP	KRRRSSKDT	LRRASVAQL	PQRATSNVF	KAEEYILKK

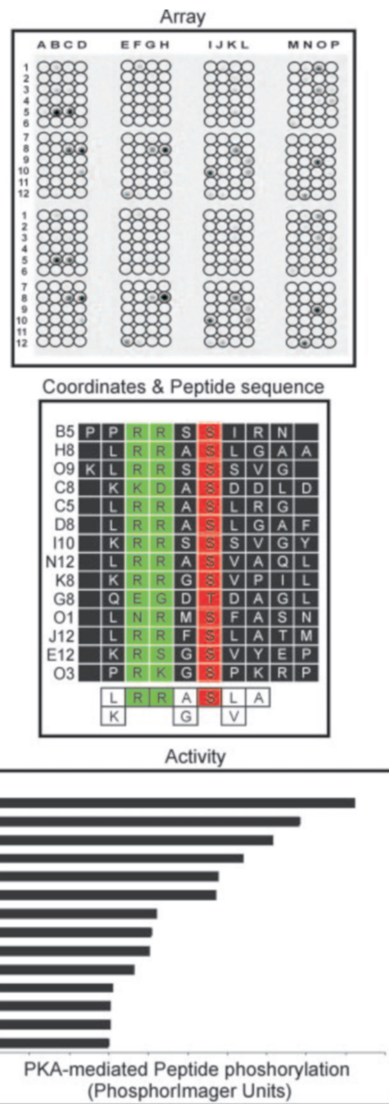

FIG. 1. **PKA phosphorylation of peptide arrays.** *Top*, overlay of the layout of the peptide array containing 2 × 192 kinase substrate peptides over the results of an *in vitro* phosphorylation of the array with purified catalytic subunit of PKA as detected by phosphorimaging and ^{33}P. In Table II the exact amino acid sequences of the peptide array are listed. The 192 pseudo-substrates are grouped in 8 clusters of 24. *Middle*, the top 12 most PKA phosphorylated peptides and a comparison to the known substrate consensus sequence for PKA are depicted. Also the location of these peptides on the array is given. *Bottom*, a quantification of the phosphorylation of the 12 most phosphorylated peptides.

A (PKA) incubation mix (500 ng/ml PKA catalytic subunit, 50 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 10% glycerol, 10 μM [γ-^{33}P]ATP, 0.01% (v/v) Brij-35, 0.01 mg/ml bovine serum albumin, ^{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 2 M NaCl, twice in demineralized H_2O, 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, 10^6 PBMCs were suspended in 1 ml of complete IMDM, and LPS stimulations were 15-min incubations (37 °C, 5% CO_2) 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. 10^6 PBMCs lysed in 200 μl of SDS sample buffer (62.5 mM Tris-HCl (pH 6.8 at

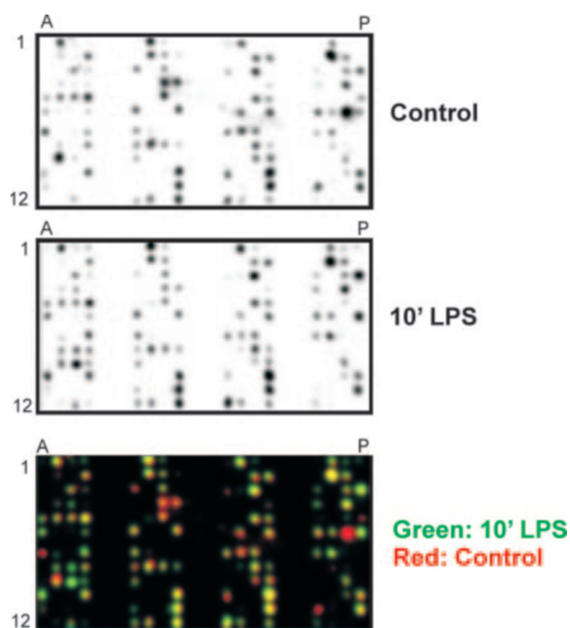

FIG. 2. **Phosphorylation of peptide arrays by cellular lysates of unstimulated cells and cells stimulated with LPS.** PBMCs were either stimulated with LPS or left untreated, which was followed by lysis of the cells and incubation of the peptide array in the presence of [γ-^{33}P]ATP. Each of the pictures represents a graphical average for better visual quality and is obtained from four different arrays out of two different experiments. Naturally, not these pictures but the original scanned pictures were used for quantification and statistical analysis of the array. The *lower picture* is an overlay in which *green dots* represent peptides that showed enhanced phosphorylation by lysates obtained from LPS-stimulated cells; *red dots* are peptides that show less phosphorylation when the lysate is stimulated with LPS, and *yellow dots* are substrates in which the phosphorylation is not altered by LPS stimulation.

25 °C), 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% (w/v) bromphenol 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-PKCζ/λ (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 9255). Non-phosphospecific actin (sc-1616) was purchased 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 chemoluminescence substrate Lumilight+ (Roche Applied Science).

Peptide Array Analysis—For kinome 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 Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM $MgCl_2$, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 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 H_2O. The cell lysates were subsequently cleared on a 0.22-μm filter. Peptide array incubation mix was produced by adding 10 μl of filter-cleared activation mix (50% glycerol, 50 μM [γ-^{33}P]ATP, 0.05% v/v Brij-35, 0.25 mg/ml bovine serum albumin, [γ-^{33}P]ATP (1000 kBq)). 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 H_2O 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.

TABLE II
LPS-upregulated kinase activity

Depicted are the effects the major substrate phosphorylating kinases in peripheral blood mononuclear cell-derived lysates on array phosphorylation of proteins from which substrate peptide sequences are derived. Results of lysates obtained with or without LPS stimulation are shown. The results represent the radioactivity incorporated in the substrates (in PhosphorImager/Aida quantification units) and are the average of four independent measurements. The average inter-spot variance of the data shown is 5%. The results presented are the 38 substrates in which phosphorylation was most up-regulated by LPS treatment. AUTO, auto-phosphorylation; JAK, janus kinase; PKC, protein kinase C; CK I, casein kinase I; MBP, myelin basic protein; PKG, protein kinase G; EIF4F, eukaryotic initiation factor 4F, GSK3, glycogen synthase kinase 3; STAT1a/b, signal transducer and activator of transcription 1a/b; PTP-2C, protein-tyrosine phosphatase 2C; TOP II, topoisomerase II; ACC, acetyl-CoA carboxylase; HSP27, heat shock protein 27; MAPKAP2, mitogen-activated protein kinase-activated protein-2; BICKS, bovine B-50-immunoreactive C-kinase substrate; AP-1, activator protein-1.

Kinase	Substrate	Control	LPS
AUTO	PKG	248	8100
JAK	STAT1a/b	248	7704
c-Src	PTP-2C	287	5960
PKA/Ca2+	Trp-5-MO	1164	23,925
Cytokine receptor	FPS	270	5512
PKA	Glycogen synthase	795	7980
PKC	alF4E	4494	41,272
PKA	v-Rel	291	2574
PKA	P450	2412	19,980
CKII	LamBR	214	1736
Cdc2	CK II	268	1728
PKA	MBP	280	1250
CKII	TOPII	231	900
PKC	ACC-a	2760	10,692
PKA	HSP27	288	1072
CKII	Lamin D	242	822
CKI	Glycogen synthase	5616	13,386
p38	MAPKAP2	10,168	21,440
Src	Annexin-2	4438	8745
p34cdc2	CK II	320	580
Glycogen synthase		9758	17,052
PKC	B1CKS	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	Tau	188	230
elF4F		21,000	25,200
p34cdc2	AP-1	460	548
Vimentin		26,240	30,889
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	Glycogen synthase	312	320
CK II	TOPII	274	280
PKA	Tau	340	340

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

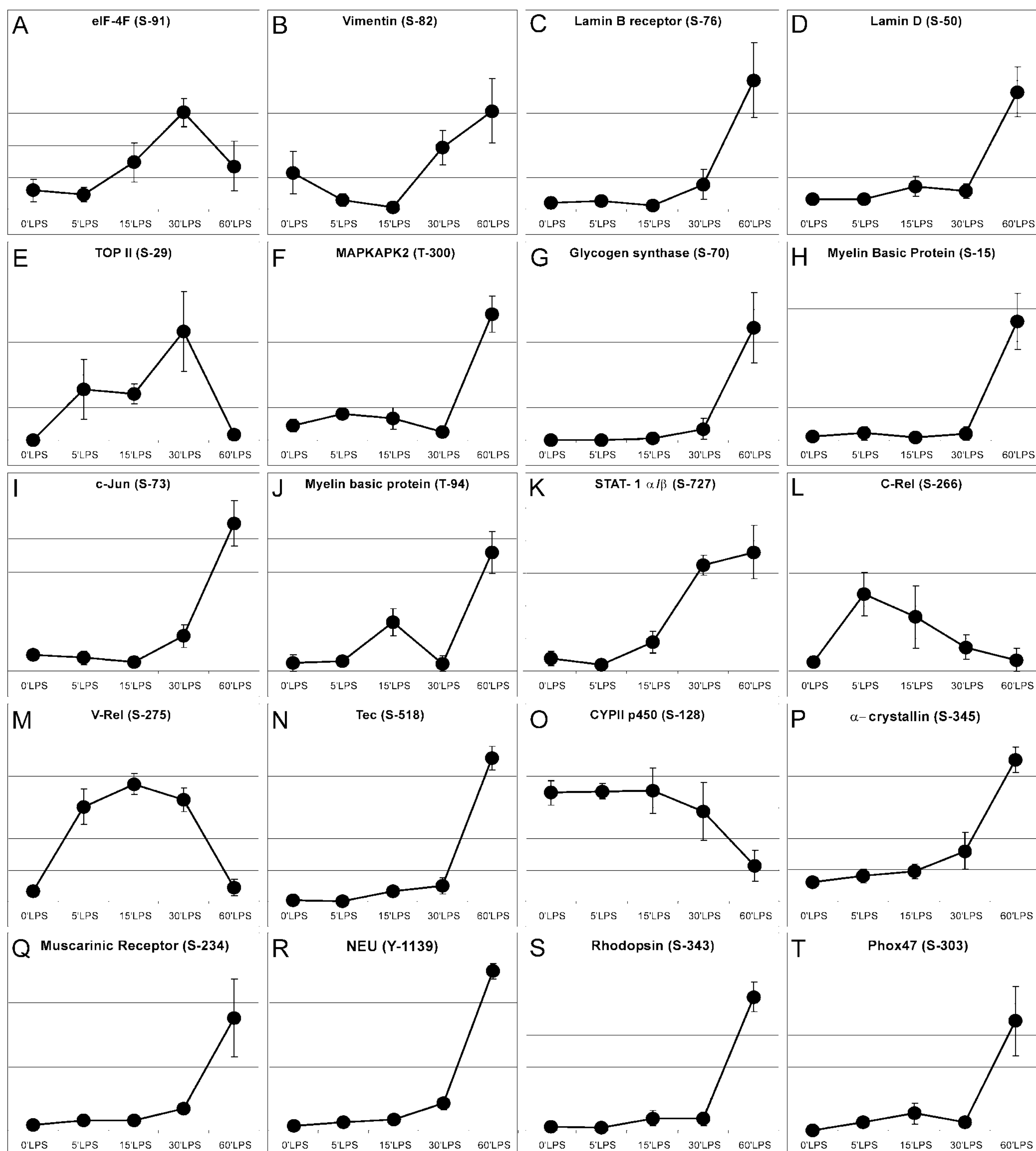

FIG. 3. **Temporal phosphorylation of different kinase substrates in PBMCs stimulated with LPS.** Peripheral blood mononuclear cells were stimulated with LPS (100 ng/ml), and lysates were obtained from the cells after 0, 5, 15, 30, and 60 min of LPS stimulation (as described under "Experimental Procedures"). 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.

(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 × 19.5 mm with each peptide spot having a diameter of ~350 μm and peptide spots being 750 μm 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 phosphoryl-

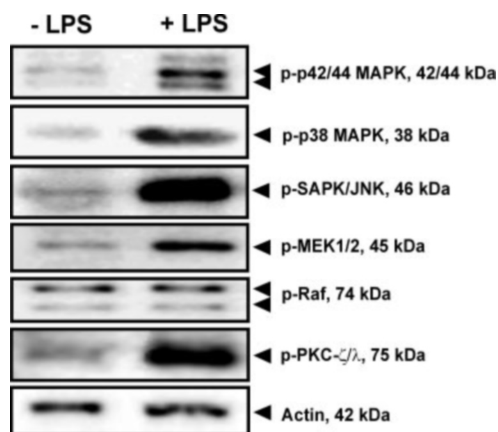

FIG. 4. **Western blot controls for LPS-induced changes in peptide phosphorylation.** The lysates for SDS-PAGE of peripheral blood mononuclear cells stimulated for 15 min with LPS or untreated cells were obtained in parallel with those used for peptide array phosphorylation, were investigated using a standard Western blotting protocol, and were probed with activation state-specific antibodies according the supplier's protocol. The apparent weight of the proteins under our conditions is indicated in the *figure*.

ation of other peptides. To test the extent to which this actually occurs, arrays were incubated for 90 min at 30 °C with 25 ng of the constitutively active catalytic subunit of PKA and [γ-^{33}P]. This treatment should result in the phosphorylation of target peptides with ^{33}P, allowing detection using phosphorimaging. As depicted in Fig. 1, this procedure resulted in strong phosphorylation of several peptides on the array. Analysis of the peptide sequence led to the extraction of the known most optimal PKA consensus sequence (17), whereas accompanying phosphorylation of peptides not containing PKA consensus phosphorylation sites was negligible (Fig. 1). Incubation of the array with PKA and [α-^{33}P]ATP did not lead to a detectable signal on the array (data not shown), demonstrating that spot phosphorylation was a specific binding of the γ-phosphate of ATP to the nonapeptides. These results identified the array as useful tool to determine substrate specificity of kinases.

Kinome Profiling of Unstimulated Human PBMCs—Next we sought to determine whether this array is also useful for kinome profiling of actual whole cell lysates. Of the oligopeptides that were phosphorylated by lysates of unstimulated human PBMCs, a significant fraction was derived from consensus sites for cytoskeletal component-derived peptides (*e.g.* vimentin lamin D and lamin B receptor) (Fig. 2, Table II), which may reflect the motile phenotype of these cells (the fraction of human PBMCs consists mainly of monocytes and T-cells). In addition, peptides derived from enzymes implicated in basal cell metabolism (*e.g.* eIF-4F, DNA topoisomerase II, and glycogen synthase) were substrates for cell lysates of these unstimulated cells (Table II). Incubation of the array with lysates and [α-^{33}P]ATP did not lead to a detectable signal on the array (data not shown), demonstrating that spot phosphorylation was not dependent on specific binding of ATP to peptides. Thus the kinome profile obtained from human PBMCs using *in vitro* phosphorylation of a peptide array is not inconsistent with the profile expected from resting mononuclear cells.

Peptide Phosphorylation by Lysates of LPS-stimulated Human PBMCs—LPS is a component of the cell wall of Gram-negative bacteria and a major activator of the innate immune system, mediating for instance septic shock in human disease, and is well documented to elicit activation of a variety of kinases in human PBMCs (18–20). We considered LPS stimulation of human PBMCs an attractive model to test array-based kinome profiling. To this end, lysates of human PBMCs were treated for 15 min with vehicle or 100 ng/ml LPS and were compared with respect to their capacity to phosphorylate peptides on arrays. The LPS stimulation resulted in the specific incorporation of ^{33}P in a variety of peptides, suggesting that the LPS treatment activated the kinases for which these peptides represent a consensus phosphorylation sequence. In this array an LPS-dependent up-regulation of phosphorylation of a variety of peptides was detected (Table II), including those with consensus phosphorylation sequences for p38 MAP kinase, p42/p44 MAP kinase, Jun-N-terminal kinase, and substrates for components of the phosphatidylinositol 3-kinase protein kinase B pathway. The validity of these signals was verified by Western blot analysis of PBMCs and subsequent probing with phosphorylation-state specific antibodies to different kinases (Fig. 3). The up-regulation of the protein kinase G autophosphorylation site was unexpected, as protein kinase G has not been implicated in LPS signaling. However, blasting this substrate against the humane genome revealed that the same substrate is also present in mitogen-activated protein kinase kinase kinase 9, and may thus actually be in strict accordance with the results obtained concerning MAP kinase activation. Thus peptide arraying seems a valid tool for studying LPS signal transduction.

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 p44/42 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 NFκB 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

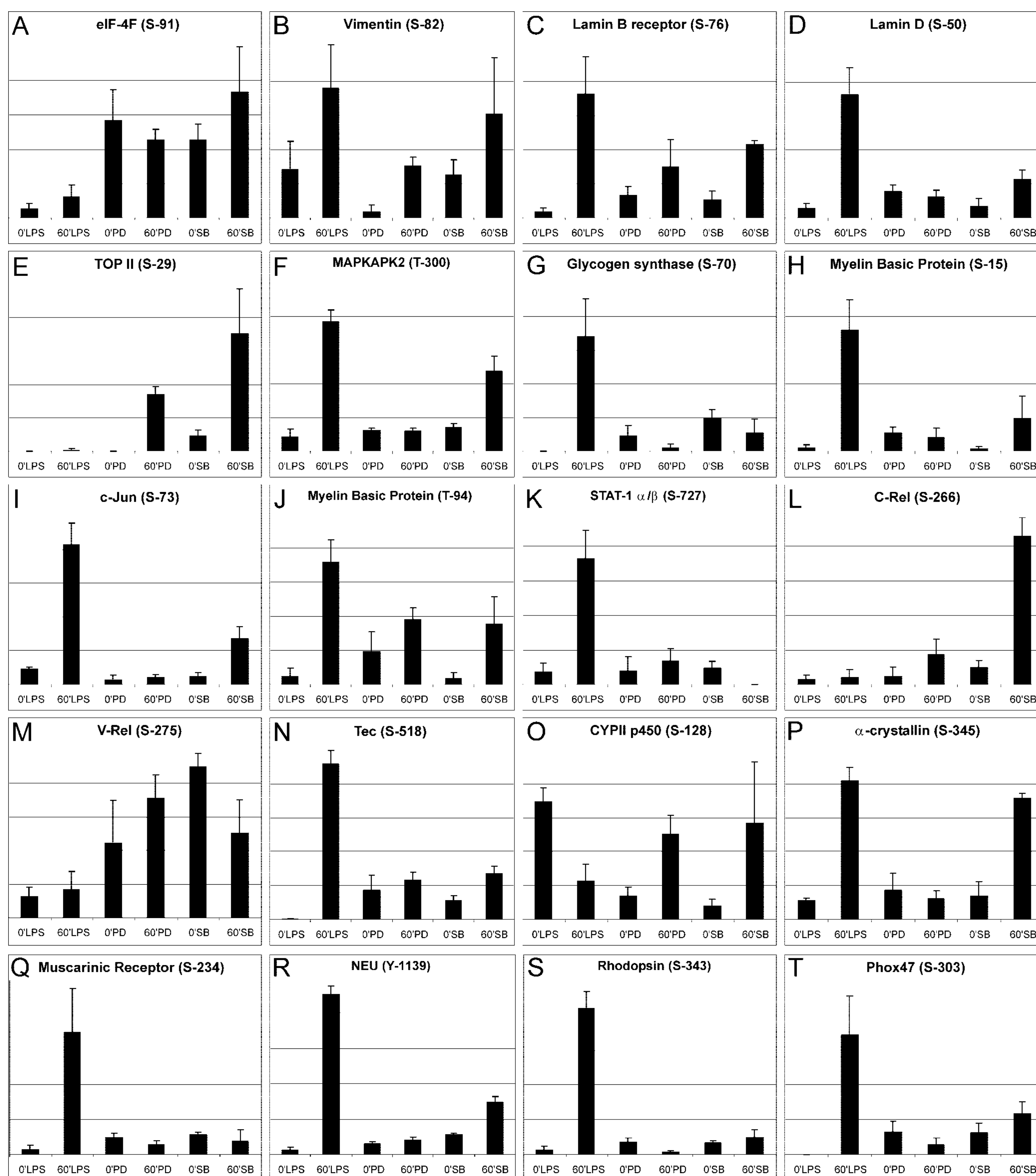

FIG. 5. **Effects of two MAP kinase inhibitors in the phosphorylation of different kinase substrates in cells stimulated with LPS.** Cells were pre-incubated for 1 h with the inhibitors PD (PD98059, 50 μM) or SB (SB203580, 10 μM) and subsequently stimulated with 100 ng/ml LPS; after 0 and 60 min the 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.

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

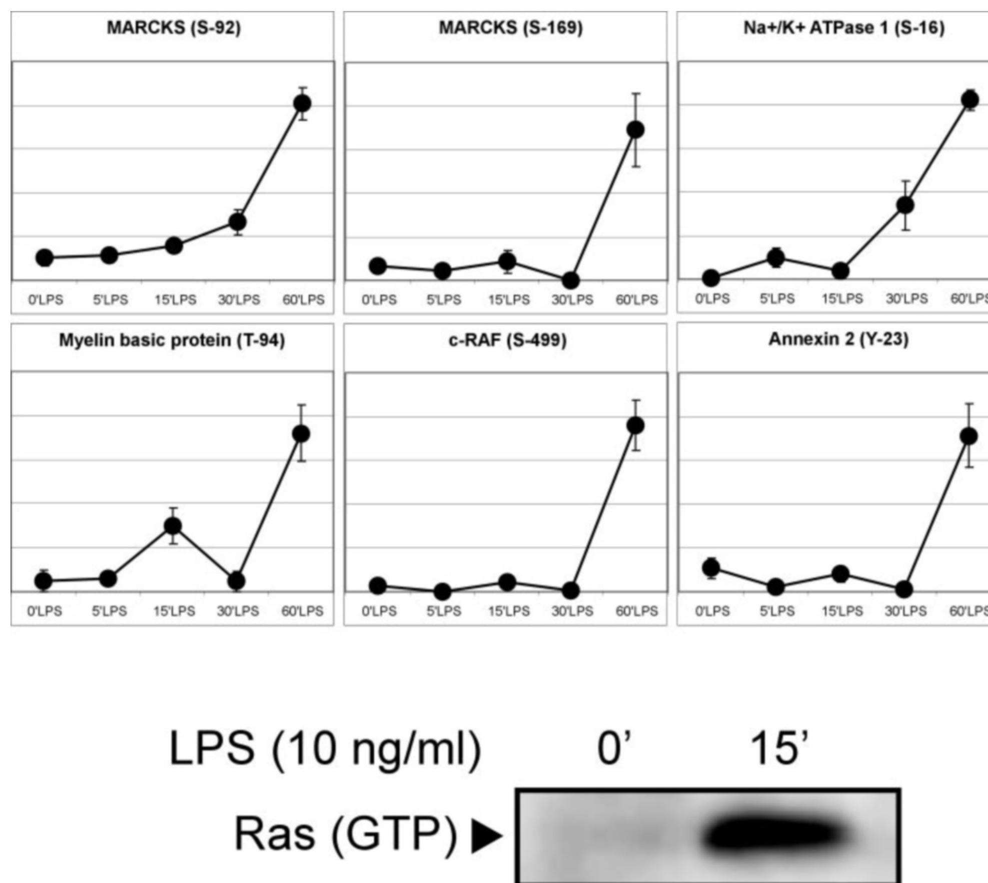

FIG. 6. **p21Ras as a target in LPS signal transduction in PBMCs.** The six graphs show a time dependent phosphorylation of different PKC and MAP kinase regulated substrates upon LPS stimulation, suggesting p21Ras involvement. A p21Ras activation assay shows elevated levels of activated p21Ras by determining the presence of p21Ras/GTP complexes in peripheral blood mononuclear cells when they are stimulated for 15 min with LPS.

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-1α/β, 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 Cβ and protein kinase Cα/β (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 (ubiquitinome), 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 kinome 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 may also 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 there are more residues inside a substrate that can be phosphorylated is also solved by using relative activation levels. Also important is that this type of array needs to be updated regularly as new and more specific substrates are being discovered.

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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