Brief Definitive Report

Association of Mitogen-activated Protein Kinases with Microtubules in Mouse Macrophages

By Aihao Ding, Bihua Chen, Michele Fuortes, and Elissa Blum

From the Beatrice and Samuel A. Seaver Laboratory, Department of Medicine, Cornell University Medical College, New York 10021

Summary

Taxol, a microtubule-binding diterpene, mimics many effects of lipopolysaccharide (LPS) on mouse macrophages. The LPS-mimetic effects of taxol appear to be under the same genetic control as responses to LPS itself. Thus we have postulated a role for microtubule-associated proteins (MAP) in the response of macrophages to LPS. Stimulation of macrophages by LPS quickly induces the activation of mitogen-activated protein kinases (MAPK). MAPK are generally considered cytosolic enzymes. Herein we report that much of the LPS-activatable pool of MAPK in primary mouse peritoneal macrophages is microtubule associated. By immunofluorescence, MAPK were localized to colchicine- and nocodazole-disruptible filaments. From both mouse brain and RAW 264.7 macrophages, MAPK could be coisolated with polymerized tubulin. Fractionation of primary macrophages into cytosol-, microfilament-, microtubule-, and intermediate filament–rich extracts revealed that ~10% of MAPK but none of MAPK kinase (MEK1 and MEK2) was microtubule bound. Exposure of macrophages to LPS did not change the proportion of MAPK bound to microtubules, but preferentially activated the microtubule-associated pool. These findings confirm the prediction that LPS activates a kinase bound to microtubules. Together with LPS-mimetic actions of taxol and the shared genetic control of responses to LPS and taxol, these results support the hypothesis that a major LPS-signaling pathway in mouse macrophages may involve activation of one or more microtubule-associated kinases.

Mitogen-activated protein kinases (MAPK), originally termed microtubule-associated protein (MAP) 2 kinase and later also called extracellular signal-regulated kinases (ERK), are rapidly activated in response to various extracellular stimuli in many cell types via a cascade (1–4) that eventuates in phosphorylation of the enzymes on both tyrosine and threonine residues (5). Substrates for MAPK are found in the nucleus, plasma membrane, cytosol, and cytoskeleton (4, 6), but it is not entirely clear which are physiologic and how MAPK, generally considered cytosolic, encounter them. Activation of MAPK by bacterial LPS (7, 8) is one of the most rapid known effects of LPS on macrophages (9) and may be required for the LPS-triggered release of eicosanoids (7) and cytokines (10, 11).

Taxol, a microtubule-binding diterpene, exerts cell cycle–independent effects on macrophages strikingly similar to those of LPS (12). These include downregulation of TNF receptors (12), activation of MAPK (9, 13), mobilization of nuclear factor (NF)-κB (14), and induction of TNF (12) and other early genes (13, 15). These responses are absent in macrophages from C3H/HeJ mice (12–15), which bear a defective allele of the *Lps* gene on chromosome 4. LPS-mimetic responsiveness to taxol cosegregated with the normal *Lps* allele in nine recombinant inbred strains (12). Inactive LPS analogues blocked taxol-induced protein tyrosine phosphorylation and expression of proinflammatory genes (16). These observations support the hypothesis that LPS and taxol share a common target in a signaling pathway controlled by the *Lps* gene (12). Although LPS and taxol may activate such a target directly from the cell surface, both enter cells rapidly (17, 18) and bind specifically to β-tubulin in a cell-free system (19–21). Thus, whether they are activated directly or indirectly, the common target of LPS and taxol may be MAP. The present report establishes that MAPK itself is one such LPS- and taxol-activated MAP.

Materials and Methods

Reagents. Anti-MAPK mAb against a 21–amino acid sequence near the COOH terminus recognizes ERK1 and ERK2 (Zymed Laboratories, Inc., South San Francisco, CA). Rabbit IgG anti-MAPK kinases (MEK1 and MEK2) was from Transduction Laboratories (Lexington, KY). Anti–α-tubulin mAb and goat antiserum against mouse vimentin were from ICN Biomedicals, Inc. (Costa Mesa, CA). Antiactin was from Miles-Yeda, Ltd. (Tel Aviv,

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/04/1899/06 $2.00
Volume 183 April 1996 1899–1904

Israel). RITC-conjugated donkey anti-mouse IgG was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). LPS prepared by phenol extraction of *Escherichia coli* serotype 0111B4 was from List Biological Laboratories, Inc. (Campbell, CA). Other reagents were as cited or from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. For immunofluorescence, thioglycollate broth–elicited peritoneal macrophages from CD-1 female mice (22) were seeded at 2×10^5 cells per 13-mm glass coverslip in 100 μl of complete medium (RPMI 1640 with 10% fetal bovine serum [FBS], 2 mM L-glutamine, 200 U/ml penicillin, and 200 μg/ml streptomycin), and nonadherent cells were removed after 2–4 h. For fractionation studies, cells were cultured in 100-mm-diameter dishes (Corning Glass, Inc., Corning, NY). For isolation of MAP, RAW 264.7 cells were maintained in spinner flasks.

Immunofluorescence Microscopy. Monolayers were fixed and permeabilized with methanol (−20°C) for 4 min. Cells were then covered with 30 μl of anti-MAPK mAb (37°C, 60 min) followed by rhodamine-conjugated donkey anti-mouse IgG (37°C, 30 min), mounted in PBS/glycerol (5:1), and examined under a fluorescence microscope (Labophot; Nikon Inc., Melville, NY).

Isolation of MAP. MAP were prepared from 10 mouse brains by the procedure of Vallee (23). In brief, tubulin in the homogenate supernate (180,000 *g*) was polymerized at 37°C by the addition of taxol and GTP, and the resulting microtubules were centrifuged through sucrose. MAP were stripped from the microtubules with 0.4 M NaCl and recovered in the 30,000 *g* supernate. By silver-stained SDS-PAGE, the MAP preparation contained one major species migrating at 220 kD and one minor species at 55 kD. These were specifically recognized by antibodies against MAP-2 and tubulin, respectively (not shown). The 30,000 *g* sediment (used below) contained only tubulin and no MAP by silver-stained SDS-PAGE and immunoblot. Similarly, $\sim 10^{10}$ RAW 264.7 cells were homogenized in 10 mM EDTA, 1 mM $MgSO_4$, pH 6.6, with 1 mM PMSF and 5 μg/ml each pepstatin A, leupeptin, aprotinin, and chymostatin, and sequentially centrifuged at 4°C (170, 30,000, and 180,000 *g*) to remove unbroken cells, nuclei, and microsomes. MAP were then isolated as for brain except that 0.6 mg purified brain tubulin was added as scaffold.

Differential Cytoskeletal Extractions. Based on the procedure of van Bergen en Henegouwen et al. (24), macrophages were washed in cytoskeleton-stabilizing buffer (CSK) containing 10 mM Pipes, pH 6.8, 250 mM sucrose, 3 mM $MgCl_2$, 150 mM KCl, 1 mM EGTA, and 1 mM PMSF and lysed at 37°C in CSK buffer containing 0.15% Triton X-100 (lysis buffer) for 5 min. Supernate S_1 (14,000 *g*, 10 min, room temperature) was considered the cytosolic fraction. $Pellet_1$ was washed three times in lysis buffer at 37°C. Microtubules were depolymerized by chilling the samples to 4°C in the same buffer and collected as S_2 (14,000 *g*, 10 min) followed by two washes with cold lysis buffer. $Pellet_2$ containing actin-based microfilaments was solubilized with 0.6 M KCl in CSK buffer in the presence of DNase (0.2 mg/ml) and $MgCl_2$ (10 mM), followed by centrifugation (14,000 *g*, 20 min), generating S_3. The remaining $pellet_3$ containing intermediate filaments was dissolved in Laemmli's sample buffer with SDS. For kinase assay, extracts were prepared in the presence of 1 mM each of sodium vanadate, sodium pyrophosphate, and sodium fluoride.

Immunoblot. Samples separated by SDS-PAGE were transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH) in an ice-water bath. Membranes were blocked in 20 mM Tris, 137 mM NaCl, pH 7.4, plus 0.1% Triton X-100 (TBST) containing 10% FBS or 5% dry milk at 4°C overnight, incubated with first antibody for 1 h at room temperature, washed three times with TBST, and incubated with horseradish peroxidase–conjugated secondary antibody (1:10,000 in TBST) for 45 min at room temperature. After extensive washing with TBST, immunoblots were developed using an enhanced chemiluminescence kit (Amersham Corp., Arlington Heights, IL).

MAPK Assay. Cell lysate was incubated with 10 μg of myelin basic protein (MBP) in kinase assay buffer (20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiothreitol, 1 mM vanadate, and 0.5 μM protein kinase A inhibitor; 25) plus 1 μCi of 5′-[γ-^{32}P]ATP (Amersham Corp.) and 50 μM cold ATP for 10 min at room temperature. The reaction was terminated by boiling in Laemmli's sample buffer. After SDS-PAGE, autoradiograms were subjected to densitometry.

Results

Localization of MAPK to Microtubules in Intact, Primary Macrophages. Anti-MAPK mAb–stained filamentous structures were unaltered by prior exposure to LPS (Fig. 1 *A*) but were dissassembled by preincubation (2 h) with the microtubule-disrupting agents colchicine (10 μM) (Fig. 1 *B*) or nocodazole (10 μM) (not shown). The pattern of MAPK distribution matched that observed with antitubulin mAb, except that anti-MAPK did not decorate microtubule-organizing centers (Fig. 1 *C*). Entirely different staining

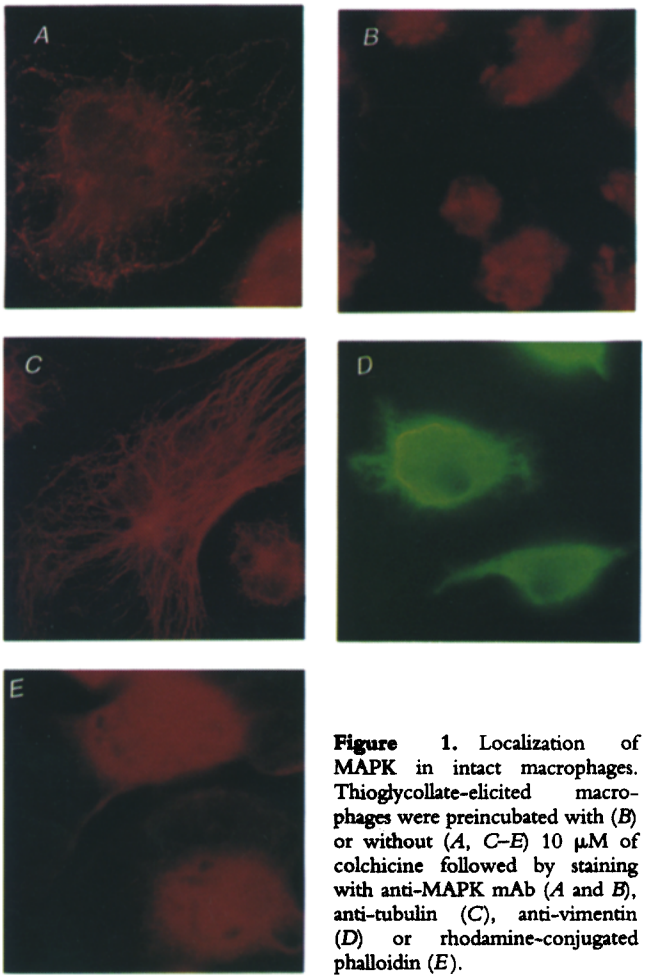

Figure 1. Localization of MAPK in intact macrophages. Thioglycollate-elicited macrophages were preincubated with (*B*) or without (*A*, *C–E*) 10 μM of colchicine followed by staining with anti-MAPK mAb (*A* and *B*), anti-tubulin (*C*), anti-vimentin (*D*) or rhodamine-conjugated phalloidin (*E*).

patterns resulted with antivimentin (marking intermediate filaments, Fig. 1 *D*) or rhodamine-conjugated phalloidin (revealing actin-rich microfilaments, Fig. 1 *E*). In contrast to the situation with growth factor–treated fibroblasts (26, 27), LPS did not cause MAPK to translocate to the macrophage nucleus.

MAPK Are among the MAP Isolated from Mouse Brain and RAW 264.7 Cells. MAP are operationally defined by their cosedimentation with polymerized microtubules through a sucrose gradient (28). MAP from mouse brain and from the macrophage-like cell line RAW 264.7 prepared in this way contained 42- and 44-kD MAPK (Fig. 2), well separated from actin (here migrating at <40 kD) and tubulin (55 kD), the identity of the latter proteins being confirmed by immunoblot. The several species binding anti–MAP-2 (Fig. 2) may correspond to known MAP-2 isoforms (29).

A Portion of MAPK Specifically Cofractionates with Microtubules in Primary Macrophages. The next experiments used a sequential extraction scheme (24) that yields fractions enriched in each of three major classes of cytoskeletal structures. About 75% of total cellular protein was recovered as cytosolic (S_1). After three washes of $pellet_1$, microtubule proteins (~7%) were recovered by depolymerization in the cold (S_2). Actin filaments (~8%) were removed by high salt extraction (S_3), and a portion of intermediate filaments remained associated with the final pellet and were extracted in SDS (S_4). Each fraction was immunoblotted with antibodies against compartment markers (Fig. 3): MAPK kinase (MEK1 and MEK2) for cytosol, tubulin for microtubules, actin for microfilaments, and vimentin for intermediate filaments. MEK was detected only in the cytosolic fraction, in contrast to observations in NIH/3T3 cells mentioned in abstract form (30). Tubulin was present in both cytosolic and microtubule fractions, consistent with the normal equilibrium between polymerized and depolymerized microtubules. Similarly, as expected, actin was both soluble and microfilament associated. Vimentin was insoluble in Triton X-100 and was only detected in the high salt extract and its SDS-soluble residue (Fig. 3).

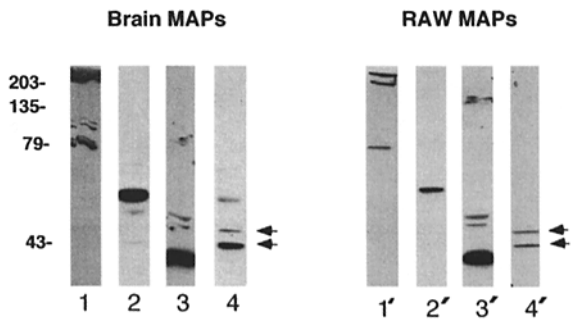

Figure 2. MAP from brain and macrophages include MAPK. Proteins cosedimenting through sucrose with polymerized tubulin were isolated from mouse brain (60 μg/lane, lanes *1–4*) and RAW 264.7 macrophages (30 μg/lane, *lanes 1′–4′*), separated on SDS-PAGE and probed with antibodies against MAP-2 (*lanes 1* and *1′*), α-tubulin (*lanes 2* and *2′*), actin (*lanes 3* and *3′*), and MAPK (*lanes 4* and *4′*). Arrows indicate positions of MAPK.

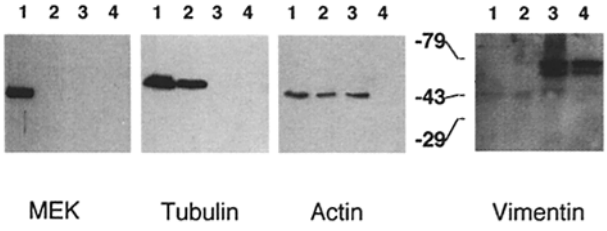

Figure 3. Sequential extraction of cytoskeletal proteins from primary macrophages. Isolated cytosolic (S_1; 5% of total recovered), microtubule- (S_2; 10%), actin- (S_3; 10%), and vimentin-containing fractions (S_4; 10%) were immunoblotted with Abs against MEK, tubulin, actin, and vimentin.

MAPK was absent in the microfilament- and intermediate filament rich fractions (Fig. 4 *A*, lanes *3* and *4*) and present in the cytosolic and microtubule-rich fractions (Fig. 4 *A*, lanes *1* and *2*). As already noted, MEK was present in cytosol but not in microtubule-rich fractions, arguing against nonspecific contamination of the latter with the former. Moreover, supernates of three washes of $pellet_1$ were analyzed to exclude the possibility that MAPK from S_1 may have associated nonspecifically with $pellet_1$ and thus persisted in S_2. No MAPK were detected in the second or third of these washes. Thus, in primary macrophages, a portion of MAPK is specifically associated with the microtubule network, but not with any other major cytoskeletal fraction. Densitometric comparison of dilutions of these fractions indicated that the microtubule-associated portion of MAPK averaged 10% of the total (8, 9, and 14% in three experiments) (Fig. 4 *B*).

LPS Preferentially Activates Microtubule-Associated MAPK. Macrophages were incubated with 0 or 100 ng/ml of LPS for 15 min, 2 h, or 18 h and subjected to sequential extraction as above. As seen in intact macrophages by immunofluorescence, LPS caused no detectable redistribution of MAPK in fractionated macrophages (Fig. 5 *A*). However, LPS (100 ng/ml, 15 min) enhanced the enzymatic activity of MAPK more markedly in the microtubule-associated fraction (3.7 ± 1.1-fold, six experiments) than in the cytosolic fraction (1.6 ± 0.3-fold) ($P < 0.015$, Student's *t* test; Fig. 5 *B*).

Discussion

Several parallel MAPK cascades regulate growth hormone–, cytokine- and stress-stimulated responses in diverse cell types (4–6, 31–36). The pleiotropic nature of MAPK action is evident from the complex array of substrates localized in different cellular compartments and executing diverse functions (31, 37–43). The postulate that MAPK must be, at least transiently, in direct contact with their physiological substrates has led to refinement of the initial description of MAPK as strictly cytosolic enzymes. In fibroblasts, mitogenic signals induce translocation of MAPK to nuclei and plasma membrane (26, 27). MAPK were found in dendritic microtubules of rat brain cells (44), in microtubule-organizing centers in mouse oocytes during

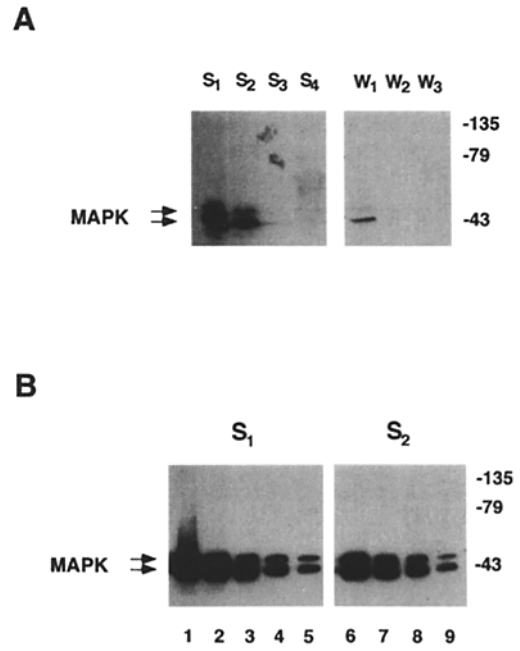

Figure 4. Association of MAPK with microtubules. (*A*) Samples of cytosolic (lane *1*), microtubule- (lane *2*), actin- (lane *3*), and vimentin (lane *4*)-containing fractions of macrophages plus the supernatants from three washes of pellet$_1$ before extraction of S_2 (lanes *5*–*7*) were probed with anti-MAPK mAb. (*B*) Semiquantitative immunoblot comparison of MAPK contents in cytosolic (S_1) and microtubule (S_2) fractions. Percentage of lysates from 4×10^7 cells loaded on the gel from S_1 were as follows: lane *1*, 2%; lane *2*, 1%; lane *3*, 0.5%; lane *4*, 0.25%; lane *5*, 0.125%; and from S_2: lane *6*, 10%; lane *7*, 5%; lane *8*, 2.5%; lane *9*, 1.25%.

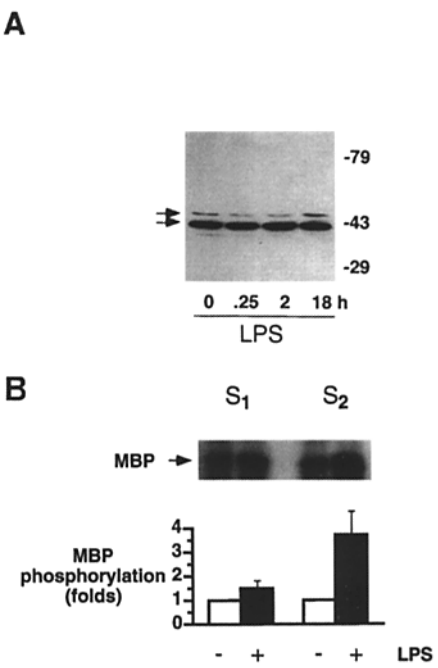

Figure 5. Effect of LPS on microtubule associated MAPK. (*A*) Primary macrophages were incubated without or with 100 ng/ml of LPS for 15 min, 2 h, or 18 h at 37°C. Microtubule-associated fractions (S_2) were extracted as in Fig. 3 and immunoblotted with anti-MAPK mAb. (*B*) Macrophages treated with or without 100 ng/ml of LPS for 15 min were sequentially extracted in the presence of phosphatase inhibitors to obtain the cytosolic (S_1) and microtubule-associated fractions (S_2). MAPK activity was assayed using MBP as a substrate in the presence of γ-[^{32}P]ATP as displayed. Autoradiogram was from one of six similar experiments. The histogram shown, in fold increase over control, is mean ± SE for all six experiments.

meiotic maturation (45), and in the microtubules of cycling mouse fibroblasts (46). To our knowledge, this report is the first to document a microtubule localization of MAPK in noncycling cells outside the nervous system, and to relate this association to responses to a microbial product.

Many MAPs, including tau, MAP-1, MAP-2, MAP-5, and caldesmon, can serve as facile substrates of MAPK (4, 47, 48). Microtubule-associated MAPK may be the physiologically relevant pool acting on MAP substrates. Phosphorylation of MAP regulates microtubule polymerization (49). Reorganization of microtubules is an important aspect of cell remodeling in such diverse situations as dendrite formation, mitosis, cell spreading, and migration.

The discovery of LPS-mimetic effects of taxol on macrophages from C3H/HeN (LPS-normoresponsive), but not C3H/HeJ (LPS-hyporesponsive) mice led to the hypothesis that these two agents might share a common target (12). LPS-mimetic effects of taxol, however, are cell cycle independent (12, 16). So far no other intracellular binding site for taxol besides polymerized tubulin has been identified. Thus, binding of taxol to microtubules may evoke two distinct signals: one leading to microtubule stabilization, the other to activation of one of LPS's signaling intermediates.

It has not been answered by what mechanism LPS and taxol activate MAPK, nor whether MAPK themselves are critical mediators of the actions of LPS and LPS-mimetic actions of taxol. Nonetheless, the microtubule association of an LPS-activatable pool of MAPK may help explain genetic and biochemical evidence that LPS and taxol share a signaling intermediate(s). It has not been excluded that other intermediates may also be activated in common by these two signals. The relevant target of MAPK or related kinases in transducing responses to LPS and taxol may itself be microtubule associated. Among other enzymes known to associate with microtubules are cAMP-dependent protein kinase (50), protein tyrosine kinase ZAP-70 (51), protein phosphatase (52), p34^{cdc2}/cyclin B complex (53), and the protooncogene products mos (54), fyn (55), and Vav (51). Indeed, MAPK can activate c-*mos* (56), and vice versa (57).

We wish to thank Dr. Carl Nathan for his support and critical reading of the manuscript.

This work was supported by National Institutes of Health grant AI-30165.

Address reprint requests to A. Ding, Cornell University Medical College, Box 57, 1300 York Avenue, New York, NY 10021.

Received for publication 24 October 1995 and in revised form 5 January 1996.

References

1. Blenis, J. 1993. Signal transduction via the MAP kinases: proceed at your own RSK. *Proc. Natl. Acad. Sci. USA.* 90:5889–5892.
2. Nishida, E., and Y. Gotoh. 1993. The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem. Sci.* 18:128–131.
3. Davis, R.J. 1994. MAPKs: new JNK expands the group. *Trends Biochem. Sci.* 19:470–473.
4. Seger, R., and E.G., Krebs. 1995. The MAPK signaling cascade. *FASEB J.* 9:726–735.
5. Anderson, N.G., J.L. Maller, N.K. Tonks, and T.W. Sturgill. 1990. Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature (Lond.).* 143:651–653.
6. David, R.J., E. Zhen, M. Cheng, S. Xu, C.A. Vanderbilt, D. Ebert, C. Garcia, A. Dang, and M.H. Cobb. 1993. Regulation and properties of extracellular signal regulated protein kinases 1, 2, and 3. *J. Am. Soc. Nephrol.* 4:1104–1110.
7. Weinstein, S.L., M.R. Gold, A.L. DeFranco. 1991. Bacterial lipopolysaccharide stimulates protein tyrosine phosphorylation in macrophages. *Proc. Natl. Acad. Sci. USA.* 88:4148–4152.
8. Weinstein, S.L., J.S. Sanghera, K. Lemke, A.L. DeFranco, and S.L. Pelech. 1992. Bacterial lipopolysaccharide induces tyrosine phosphorylation and activation of mitogen-activated protein kinases in macrophages. *J. Biol. Chem.* 267:14955–14962.
9. Ding, A., E. Sanchez, and C.F. Nathan. 1993. Taxol shares the ability of bacterial lipopolysaccharide to induce tyrosine phosphorylation of microtubule-associated protein kinase. *J. Immunol.* 151:1–7.
10. Geppert, T.D., C.E. Whitehurst, P. Thompson, and B. Beutler. 1994. Lipopolysaccharide signals activation of tumor necrosis factor biosynthesis through the Ras/Raf-1/MEK/MAPK pathway. *Mol. Med.* 1:93–103.
11. Reimann, T., D. Buscher, R.A. Hipskind, S. Krautwald, M.-L. Lohmann-Matthes and M. Baccarini. 1994. Lipopolysaccharide induces activation of the Ref-1/MAP kinase pathway. *J. Immunol.* 153:5740–5749.
12. Ding, A., F. Porteu, E. Sanchez, and C.F. Nathan. 1990. Shared actions of endotoxin and taxol on TNF receptors and TNF release. *Science (Wash. DC).* 248:370–372.
13. Manthey, C.L., M.E. Brandes, P.Y. Perera, and S.N. Vogel. 1992. Taxol increases steady-state levels of lipopolysaccharide-inducible genes and protein-tyrosine phosphorylation in murine macrophages. *J. Immunol.* 149:2459–2465.
14. Hwang, S., and A. Ding. 1995. Activation of NF-κB in murine macrophages by taxol. *Cancer Biochem. Biophys.* 14:265–272.
15. Bogdan, C., and A. Ding. 1992. Taxol, a microtubule-stabilizing antineoplastic agent, induces expression of tumor necrosis factor alpha and interleukin-1 in macrophages. *J. Leukoc. Biol.* 52:119–121.
16. Manthey, C.L., N. Qureshi, P.L. Stutz, and S.N. Vogel. 1993. Lipopolysaccharide antagonists block taxol-induced signaling in murine macrophages. *J. Exp. Med.* 178:695–701.
17. Kang, Y.-H., R.S. Dwivedi, and C.-H. Lee. 1990. Ultrastructural and immunocytochemical study of uptake and distribution of bacterial lipopolysaccharide in human monocytes. *J. Leukoc. Biol.* 48:316–332.
18. Balasubramanian, S.V., and R.M. Straubinger. 1994. Taxol-lipid interactions: taxol-dependent effects on the physical properties of model membranes. *Biochemistry.* 33:8941–8947.
19. Ding, A., E. Sanchez, M. Tancino, and C.F. Nathan. 1992. Interactions of bacterial lipopolysaccharides with microtubule proteins. *J. Immunol.* 148:2853–2858.
20. Rao, S., N.E. Krauss, J.M. Heerding, C.S. Swindell, I. Ringel, G.A. Orr, and S.B. Horwitz. 1994. 3′-(p-azidobenzamido)taxol photolabels the N-terminal 31 amino acids of β-tubulin. *J. Biol. Chem.* 269:3132–3134.
21. Rao, S., G.A. Orr, A.G. Chaudhary, D.G.I. Kingston, and S.B. Horwitz. 1995. Characterization of the taxol binding site on the microtubule. *J. Biol. Chem.* 270:20235–20238.
22. Ding, A., C.F. Nathan, and D.J. Stuehr. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J. Immunol.* 141:2407–2412.
23. Vallee, R.B. 1986. Purification of brain microtubules and microtubule associated protein 1 using taxol. *Methods Enzymol.* 134:104–115.
24. van Bergen en Henegouwen, P.M.P., J.C. den Hartigh, P. Romeyn, A.J. Verkleij, and J. Boonstra. 1992. The epidermal growth factor receptor is associated with actin filaments. *Exp. Cell Res.* 199:90–97.
25. Glove, J.R., D.J. Price, H.M. Goodman, and A. Avruch. 1987. Recombinant fragment of protein kinase inhibitor blocks cyclic AMP-dependent gene transcription. *Science (Wash. DC).* 238:530–533.
26. Gonzalez, F.A., A. Seth, D.L. Raden, D.S. Bowman, and F.S. Fay. 1993. Serum-induced translocation of mitogen-activated protein kinase to the cell surface ruffling membrane and the nucleus. *J. Cell. Biol.* 122:1089–1101.
27. Lenormand, P., C. Sardet, G. Pagès, G. L'Allemein, A. Brunet, and J. Pouysségur. 1993. Growth factors induce nuclear translocation of MAP kinases ($p42^{mapk}$ and $p44^{mapk}$) but not of their activator MAP kinase kinase ($p45^{mapkk}$) in fibroblasts. *J. Cell Biol.* 122:1079–1088.
28. Olmsted, J.B. 1986. Microtubule associated-proteins. *Annu. Rev. Cell Biol.* 2:421–457.
29. Olesen, O.F., 1994. Expression of low molecular weight isoforms of microtubule-associated protein 2. *J. Biol. Chem.* 269: 32904–32908.
30. Reszka, A.A., R. Seger, C.D. Diltz, E.G. Krebs, and E.H. Fischer. 1995. Association of mitogen-activated protein kinase (MAPK) and kinase kinase (MAPKK) with the cytoskeleton. *J. Cell. Biochem.* 19B:134, J4–309. (Abstr.)
31. Sturgill, T.W., L.B. Ray, E. Erikson, J. Maller. 1988. Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature (Lond.).* 334:715–718.
32. Ahn, N.G., and E.G. Krebs. 1990. Evidence for an epidermal growth factor-stimulated protein kinase cascade in Swiss 3T3

cells. Activation of serine peptide kinase activity by myelin basic protein kinases in vitro. *J. Biol. Chem.* 265:11495–11501.

33. Ahn, N.G., R. Seger, R.L. Bratlien, and E.G. Krebs. 1992. Growth factor-stimulated phosphorylation cascades: activation of growth factor-stimulated MAP kinase. *Ciba Found. Symp.* 164:126–131.
34. Qui, M.S., and S. H. Green. 1992. PC12 cell neuronal differentiation is associated with prolonged $p21^{ras}$ activity and consequent prolonged ERK activity. *Neuron.* 9:705–717.
35. Alberola-Ila, J., K.A. Forbush, R. Seger, E.G. Krebs, and R.M. Pulmutter. 1995. Selective requirement for MAP kinase activation in thymocyte differentiation. *Nature (Lond.)* 373:620–623.
36. Tsai, M., R.H. Chen, S.Y. Tam, J. Blenis, and S.J. Galli. 1993. Activation of MAP kinases, $pp90^{rsk}$ and pp70-S6 kinases in mouse mast cells by signaling through the c-kit receptor tyrosine kinase or Fc epsilon RI: rapamycin inhibits activation of pp70-S6 kinases and proliferation in mouse mast cells. *Eur. J. Immunol.* 23:3286–3291.
37. Takishima, K., I. Griswold-Prenner, T. Ingebritsen, and M.R. Rosner. 1991. Epidermal growth factor (EGF) receptor T669 peptide kinase from 3T3-L1 cells is an EGF-stimulated "MAP" kinase. *Proc. Natl. Acad. Sci. USA.* 88:2520–2524.
38. Northwood, I.C., F.A. Gonzalez, M. Wartmann, D.L. Raden, and R.J. Davis. 1991. Isolation and characterization of two growth factor-stimulated protein kinases that phosphorylate the epidermal growth factor receptor at threonine 669. *J. Biol. Chem.* 266: 15266–15276.
39. Pulverer, B.J., J.M. Kyriakis, J. Avruch, E. Nikolakaki, and J.R. Woodgett. 1991. Phosphorylation of c-jun mediated by MAP kinases. *Nature (Lond.)* 353:670–674.
40. Alvarez, E., I.C. Nothwood, and F.A. Gonzalez. 1991. Pro-Leu-Ser.Thr-Pro is a consensus primary sequence for substrate protein phosphorylation. Characterization of the phosphorylation of c-myc and c-jun proteins by an epidermal growth factor receptor threonine 669 protein kinase. *J. Biol. Chem.* 266:15277–15285.
41. Drechsel, D.N., A.A. Hyman, M.H. Cobb, and M.W. Kirschner. 1992. Modulation of the dynamic instability of tubulin assembly by the microtubule-associated protein tau. *Mol. Biol. Cell.* 3:1141–1154.
42. Gotoh, Y., E. Yoshida, and S. Matsuda. 1991. *In vitro* effects on microtubule dynamics of purified *Xenopus* M phase-activated MAP kinase. *Nature (Lond.).* 349:251–254.
43. Tsao, H., J.M. Aletta, and L.A. Greene. 1990. Nerve growth factor and fibroblast growth factor selectively activate a protein kinase that phosphorylates high molecular weight microtubule-associated proteins. Detection, partial purification, and characterization in PC12 cells. *J. Biol. Chem.* 265:15471–15480.
44. Fiore, R.S., V.E. Bayer, S.L. Pelech, J. Posada, J.A. Cooper, and J.M. Baraban. 1993. p42 mitogen-activated protein kinase in brain prominent localization in neuronal cell bodies and dendrites. *Neuroscience.* 55:463–472.
45. Verlhac, M.H., H. de Pennart, B. Maro, M.H. Cobb, and H.J. Clarke. 1993. MAP kinase becomes stably activated at metaphase and is associated with microtubule-organizing centers during meiotic maturation of mouse oocytes. *Dev. Biol.* 158:330–440.
46. Reszka, A.A., R. Seger, C.D. Diltz, E.G. Krebs, and E.H. Fischer. 1995. Association of mitogen-activated protein kinase with the microtubule cytoskeleton. *Proc. Natl. Acad. Sci. USA.* 92:8881–8885.
47. Hoshi, M., K. Ohta, Y. Gotoh, A. Mori, H. Murofushi, H. Sakai, and E. Nishida. 1992. Mitogen-activated-protein-kinase-catalyzed phosphorylation of microtubule-associated proteins, microtubule-associated protein 2 and microtubule-associated protein 4, induces an alteration in their function. *Eur. J. Biochem.* 203:43–52.
48. Childs, T.J., M.H. Watson, J.S. Sanghera, D.L. Campbell, S.L. Pelech, and A.S. Mak. 1992. Phosphorylation of smooth muscle caldesmon by mitogen-activated protein (MAP) kinase and expression of MAP kinase in differentiated smooth muscle cells. *J. Biol. Chem.* 267:22853–22859.
49. Jameson, L., and M. Caplow. 1981. Modification of microtubule steady-state dynamics by phosphorylation of the microtubule-associated proteins. *Proc. Natl. Acad. Sci. USA.* 78: 3413–3417.
50. Perez, J., D. Tinelli, C. Cagnoli, P. Pecin, N. Brunello, and G. Racagni. 1993. Evidence for the existence of cAMP-dependent protein kinase phosphorylation system associated with specific phosphoproteins in stable microtubules from rat cerebral cortex. *Brain Res.* 602:77–83.
51. Huby, R.D.J., G.W. Carlile, and S.C. Ley. 1995. Interactions between the protein-tyrosine kinase ZAP-70, the proto-oncoprotein Vav, and tubulin in Jurkat T cells. *J. Biol. Chem.* 270:30241–30244.
52. Sontag, E., V. Nunbhakdi-Craig, G.S. Bloom, and M.C. Mumby. 1995. A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. *J. Cell Biol.* 128:1131–1144.
53. Ookata, K., S.-I. Hisanaga, J.C. Bulinski, H. Murofushi, H. Aizawa, T.J. Itoh, H. Hotani, E. Okumura, K. Tachibana, and T. Kishimoto. 1995. Cyclin B interaction with microtubule-associated protein 4 (MAP4) targets $p34^{cdc2}$ kinase to microtubules and is a potential regulator of M-phase microtubule dynamics. *J. Cell Biol.* 128:849–862.
54. Zhou, R., M. Oskarsson, R.S. Paules, N. Schulz, D. Cleveland, and G.F. vande Woude. 1991. Ability of the c-mos product to associate with and phosphorylate tubulin. *Science (Wash. DC).* 251:671–678.
55. Ley, S.C., M. Marsh, C.R. Bebbington, K. Proundfoot, and P. Jordan. 1994. Distinct intracellular localization of Lck and Fyn protein tyrosine kinases in human T lymphocytes. *J. Cell Biol.* 125:639–649.
56. Haccard, O., B. Sarcevic, A. Lewellyn, R. Hartley, L. Roy, T. Izumi, E. Erikson, and J.L. Maller. 1993. Induction of metaphase arrest in cleaving *Xenopus* embryos by MAP kinase. *Science (Wash. DC).* 262:1262–1265.
57. Shibuya, E.K., and J.V. Ruderman. 1993. Mos induces the in vitro activation of mitogen-activated protein kinases in lysates of frog oocytes and mammalian somatic cells. *Mol. Biol. Cell.* 4:781–790.