Association of Mitogen-activated Protein Kinases with Microtubules in Mouse Macrophages
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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 anti–mouse vimentin were from ICN Biomedicals, Inc. (Costa Mesa, CA). Antiactin was from Miles-Yeda, Ltd. (Tel Aviv,
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 x 10^6 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). A different antibody was used to determine the presence of MAPK in macronuclei.

**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, ∼10^10 RAW 264.7 cells were homogenized in 10 mM EDTA, 1 mM MgSO₄, 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 Ex 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 MgCl₂, 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 S₁ (14,000 g, 10 min, room temperature) was considered the cytosolic fraction. Pellet₁ 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 S₂ (14,000 g, 10 min) followed by two washes with cold lysis buffer. Pellet₂ containing actin-based microfilaments was solubilized with 0.6 M KCl in CSK buffer in the presence of DNase (0.2 mg/ml) and MgCl₂ (10 mM), followed by centrifugation (14,000 g, 20 min), generating S₃. The remaining pellet, 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 peroxi-

**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 MgCl₂, 2 mM EGTA, 1 mM dithiothreitol, 1 mM vanadate, and 0.5 μM protein kinase A inhibitor; 25) plus 1 μCi of 32P-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 disassembled by preincubation (2 h) with the microtubule-disrupting agents colchicine (10 mM) (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. 1D) or rhodamine-conjugated phalloidin (revealing actin-rich microfilaments, Fig. 1E). 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. MAPK 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 (S1). After three washes of pellet, microtubule proteins (~7%) were recovered by depolymerization in the cold (S2). Actin filaments (~8%) were removed by high salt extraction (S3), and a portion of intermediate filaments remained associated with the final pellet and were extracted in SDS (S4). 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).

MAPK was absent in the microfilament- and intermediate filament rich fractions (Fig. 4A, lanes 3 and 4) and present in the cytosolic and microtubule-rich fractions (Fig. 4A, 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 were analyzed to exclude the possibility that MAPK from S1 may have associated nonspecifically with pellet1 and thus persisted in S2. 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. 4B).

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. 5A). 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. 5B).

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
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^cdk2/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).

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