Dynamin Is Required for the Activation of Mitogen-activated Protein (MAP) Kinase by MAP Kinase Kinase

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Internalization of activated receptors from the plasma membrane has been implicated in the activation of mitogen-activated protein (MAP) kinase. However, the mechanism whereby membrane trafficking may regulate mitogenic signaling remains unclear. Here we report that dominant-negative dynamin (K44A), an inhibitor of endocytic vesicle formation, abrogates MAP kinase activation in response to epidermal growth factor, lysophosphatidic acid, and protein kinase C-activating phorbol ester. In contrast, dynamin-K44A does not affect the activation of Ras, Raf, and MAP kinase kinase (MEK) by either agonist. Through immunofluorescence and subcellular fractionation studies, we find that activated MEK is present both at the plasma membrane and in intracellular vesicles but not in the cytosol. Our findings suggest that dynamin-regulated endocytosis of activated MEK, rather than activated receptors, is a critical event in the MAP kinase activation cascade.

The mitogen-activated protein (MAP) kinase family is highly conserved serine/threonine protein kinases that are activated by diverse extracellular stimuli and mediate a wide variety of cellular responses (1–3). The p42/p44 MAP kinases, also named extracellular-regulated kinases (ERKs), function in a signaling cascade from the plasma membrane to the nucleus that controls cell cycle progression and differentiation and, furthermore, plays a key role in oncogenic transformation (1–3). Mitogens such as epidermal growth factor (EGF) and lysophosphatidic acid (LPA) trigger the MAP kinase cascade through activation of Ras via recruitment of the guanine nucleotide exchange factor Sos. Protein kinase C (PKC)-activating phorbol ester can also trigger the Ras-MAP kinase pathway, but the mechanism is distinct from that initiated by cell-surface receptors and presumably involves decreased Ras-GAP activity (4, 5). Active Ras binds to and activates the protein kinase c-Raf-1 (referred to as Raf), Raf then phosphorylates and thereby activates the cytosolic MAP kinase kinases, MEK1 and MEK2, which in turn activate the p42/p44 MAP kinases (ERK1 and ERK2) by dual phosphorylation on threonine and tyrosine. Whereas Ras-Raf interaction takes place at the plasma membrane, it remains unclear where MEK activation occurs, at the membrane or in the cytoplasm. Activated MEK remains in the cytoplasm (6, 7), whereas activated MAP kinases can undergo translocation to the nucleus where they modulate gene expression through phosphorylation of transcription factors (1–3, 6, 8–12).

Recent evidence points to an essential, though poorly understood, role of receptor endocytosis in the activation of MAP kinase (13–19). Receptor endocytosis is regulated by dynamin, a 100 kDa GTPase that is targeted to clathrin-coated pits where it oligomerizes around the neck of budding vesicles (reviewed in Ref. 20), although its precise mode of action remains to be elucidated (Refs. 21 and 22, and references therein). Dynamin with a point mutation in the nucleotide-binding site (K44A) interferes with the function of endogenous dynamin by blocking vesicle internalization before membrane scission occurs (23). Expression of dynamin-K44A inhibits MAP kinase activation by receptor tyrosine kinases, such as those for EGF, insulin, and insulin-like growth factor (13–15) and by G protein-coupled receptor agonists, including LPA, isoproterenol, thrombin, opioids, and serotonin (16–19). Moreover, we and others recently identified endogenous dynamin as a component in the pathway that links LPA and β-adrenergic receptors to MAP kinase activation (24, 25). These findings have led to the notion that dynamin-regulated internalization of activated receptors is obligatory for MAP kinase activation (16–18). Yet, the emerging picture is far from clear. For example, endocytosis-defective mutant EGF receptors show enhanced rather than reduced mitogenicity (26), whereas an endocytosis-defective Gαs-coupled muscarinic receptor can still activate MAP kinase in an identical manner to wild-type receptor (27). In fact, there is no direct evidence that internalization of the activated receptor itself is necessary for MAP kinase activation; in principle, dynamin-controlled endocytosis might regulate any step along the route from cell-surface receptor to MAP kinase.

Here we have examined the role of dynamin in activation of the Ras-MAP kinase pathway by EGF, LPA, and phorbol ester. In particular, we address the question of where dynamin acts in the pathway from activated receptor to MAP kinase. We show that dynamin function is essential for MAP kinase activation by activated MEK, but not for activation of Ras, Raf, or MEK, in response to all agonists tested. We conclude that dynamin-regulated endocytosis of activated MEK, rather than activated receptors, is critical for MAP kinase activation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium containing 7.5% fetal calf serum and antibiotics. Cells were cDNA transfected using the DEAE method as described previously (28). Transfection efficiency was as high as 70–80%. The pMT2-dynamin-1 (WT and K44A) constructs were generated by ligating the respective cDNAs (from Dr. S. Schmid, The Scripps Research Institute, CA) into pMT2 using EcoRI/XbaI. The HA-tagged dynamin-2 cDNAs (from Dr. S. Schmid, The Scripps Research Institute, CA) were cloned into pcDNA3 using EcoRI/XhoI. Plasmids pMT2-Myc-ERK2 and
cells were lysed in a buffer containing 20 mM Hepes, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 5 mM MgCl₂, and 10% glycerol. The lysates were incubated with 20 μM of GST-Raf(RBD) (expression construct provided by Dr. J. Bos, Laboratory for Physiological Chemistry, The Netherlands) and were washed three times in lysis buffer. The amount of Ras pulled-down was then assessed by Western blotting using anti-Ras antibody (no. R02120; Transduction Labs). After serum starvation, cells were stimulated with EGF (2.5 ng/ml, 5 min) and Ras activation was assessed using GST-Raf(RBD) pull-down followed by anti-Ras Western blotting. (The slightly elevated levels of activated Ras in dynamin-K44A-expressing cells were consistently observed and are possibly because of accumulation of upstream activators at the plasma membrane). Molecular weight markers (kDa) are indicated on the left.

**Immunoprecipitation and Western Blotting**—After stimulation with either LPA (1 μM), EGF (5 ng/ml), or TPA (200 nm) for 5 min. Activity of immunoprecipitated Myc-ERK2 was measured using myelin basic protein as a substrate. Error bars represent S.D. (n = 6). Equal expression levels of Myc-ERK2 and dynamin-1-K44A were confirmed by Western blotting of total lysates (lower panel).

**Ras Activation Assay**—Cells were stimulated and washed as described above. Ras activation was assayed as described (30). In brief, cells were lysed in a buffer containing 20 mM Hepes, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 5 mM MgCl₂, and 10% glycerol. The lysates were incubated with 20 μM of GST-Raf(RBD) (expression construct provided by Dr. J. Bos, Laboratory for Physiological Chemistry, The Netherlands) and were washed three times in lysis buffer. The amount of Ras pulled-down was then assessed by Western blotting using anti-Ras antibody (no. R02120; Transduction Labs). In all pull-down experiments, the lysates were pre-cleared using GST. Analysis of Ras binding to GST alone was performed in each assay but was not detected, indicating that Ras binds to GST-RBD in an RBD- and stimulus-dependent manner.

**In Vitro Kinase Assays**—The activity of transfected Myc-tagged ERK2 was assessed by anti-Myc (9E10) immunoprecipitation and a subsequent in vitro kinase reaction as described previously (28). Expression of Myc-ERK2 and co-transfected K44A-dynamin-1 or WT-dynamin-2 was assessed by Western blotting of total lysates. The activity of Raf was tested by immunoprecipitation using antibody R19120 (Transduction Labs) and a subsequent in vitro kinase assay as described (31). In all kinase assays, pre-clears were performed with either normal mouse serum (and rabbit anti-mouse) or normal rabbit serum in

**FIG. 1. Inhibition of MAP kinase activation by dynamin-K44A.** Serum-starved COS-7 cells transfected with Myc-ERK2, a control vector, or a vector encoding dynamin-1-K44A were stimulated with LPA (1 μM), EGF (2.5 ng/ml), or TPA (200 nm) for 5 min. Activity of immunoprecipitated Myc-ERK2 was measured using myelin basic protein as a substrate. Error bars represent S.D. (n = 6). Equal expression levels of Myc-ERK2 and dynamin-1-K44A were confirmed by Western blotting of total lysates (lower panel).

**FIG. 2. Differential effects of genistein on activation of Ras and MAP kinase by LPA, EGF, and TPA.** Serum-starved COS-7 cells were either left untreated or were pre-incubated with genistein (50 μM, 30 min) prior to stimulation with LPA, EGF, or TPA. A. Ras activation, measured by GST-Raf(RBD) pull-down followed by anti-Ras Western blotting. No Ras was detected in control GST pull-down experiments (not shown). B. MAP kinase activation as assessed by Western blotting of total cell lysates using antibody against phosphorylated ERK1 and ERK2 (pMAPK). Molecular mass markers (kDa) are shown on the left.

**FIG. 3. Dynamin-K44A does not interfere with Ras activation.** COS-7 cells were transfected with empty control vector, dynamin-K44A or activated Gα12 (Q → L mutation). After serum starvation, cells were stimulated with EGF (2.5 ng/ml, 5 min) and Ras activation was assessed using GST-Raf(RBD) pull-down followed by anti-Ras Western blotting. (The slightly elevated levels of activated Ras in dynamin-K44A-expressing cells were consistently observed and are possibly because of accumulation of upstream activators at the plasma membrane). Molecular weight markers (kDa) are indicated on the left.

**FIG. 4. Effects of dynamin-K44A and WT-dynamin on the activation of Raf, MEK, and MAP kinase.** A. dynamin-K44A inhibits MAP kinase activation but not Raf or MEK activation. Cells transfected with empty vector or dynamin-1-K44A were stimulated with either EGF or TPA (5 min). Raf activity was determined by an in vitro kinase assay. Total lysates were analyzed for the presence of activated MEK (pMEK) and MAP kinase (pMAPK) using phospho-specific antibodies. B. WT-dynamin potentiates MAP kinase activation but not MEK activation. Cells transfected with Myc-tagged ERK2 together with control vector, WT-dynamin-1, or dynamin-1-K44A were stimulated with the indicated agonists. Left panel, activity of immunoprecipitated Myc-ERK2, as determined by an in vitro kinase assay as in Fig. 1. Right panel, activation of endogenous MAP kinase and MEK measured in control cells and in cells expressing WT-dynamin-1 using phospho-specific antibodies. C. effects of dynamin-2. Cells transfected with empty vector, WT-dynamin-2, or K44A-dynamin-2 were stimulated with EGF. Activation of endogenous MEK and MAP kinase was assessed by blotting with phospho-specific antibodies as in panel A. Molecular mass markers (kDa) are indicated on the left.

In all kinase assays, pre-clears were performed with either normal mouse serum (and rabbit anti-mouse) or normal rabbit serum in
MEK is localized to the plasma membrane and in intracellular vesicles. The Cells were processed for immunofluorescence using an antibody against activated (phospho)-MEK. Confocal microscopy reveals that activated structures.

Subcellular Fractionation—After agonist stimulation, cells were washed once with ice-cold PBS and subsequently scraped in a buffer containing 50 mM Tris, pH7.4, 150 mM NaCl, 1 mM EGTA, supplemented with protease inhibitors. The cells were then sonicated with 60 1-s pulses. Undisrupted cells were cleared by a short spin (2 min at 3000 rpm; Eppendorf table centrifuge). The supernatant was then ultracentrifuged for 1h at 100,000 x g; the supernatant contains cytosol, whereas the pellet (p100 fraction) contains membranes and cytoskeletal structures.

RESULTS AND DISCUSSION

Dynamin Function Is Required for MAP Kinase Activation by EGF, LPA, and TPA—We set out to examine the role of dynamin in MAP kinase activation using monkey kidney COS-7 cells. In these cells, the Ras-MAP kinase cascade is strongly activated by three distinct agonists: (i) EGF which signals via its tyrosine kinase receptor ErbB-1; (ii) LPA, the prototypic G protein-coupled receptor ligand that activates Ras through Gi (32–34); and (iii) PKC-activating phorbol ester TPA, which activates Ras in a Sos-independent manner (5). Fig. 1 shows that EGF, LPA, and TPA induce a 10- to 20-fold increase in MAP kinase (ERK2) activity, as measured by immunoprecipitation of transfected Myc-ERK2 and subsequent in vitro kinase assays. Essentially similar results were obtained in blotting assays using an antibody against active endogenous MAP kinase (see below).

Expression of dominant-negative dynamin-K44A (using either the dynamin-1 or dynamin-2 isoform; see below) results in almost complete inhibition of MAP kinase activity induced by both EGF and LPA (Fig. 1). Surprisingly, dynamin-K44A also inhibits MAP kinase activation in response to TPA which bypasses cell-surface receptors. We considered the formal possibility that TPA might signal through transactivation of a receptor tyrosine kinase (35, 36). This possibility is ruled out, however, because the tyrosine kinase inhibitor genistein abrogates EGF- and LPA-induced activation of both Ras and MAP kinase (28, 32, 33) without affecting the responses to TPA (Fig. 2, A and B). Similarly, the tyrosine kinase inhibitor amino-(methylphenyl)-(t-butyl)pyrazolo-pyrimidine (PP1; 10 μM) abolished EGF- and LPA-induced, but not TPA-induced, MAP kinase activation (data not shown). These results indicate that the inhibitory effect of dynamin-K44A on MAP kinase activation is not simply attributable to impaired receptor internalization.

Activation of Ras, Raf, and MEK Is Independent of Dynamin Function—The above findings suggest that dynamin is required either at the level of Ras-GTP accumulation or at a step downstream of activated Ras, or both. We therefore tested the effect of dynamin-K44A on activation of Ras, Raf, and MEK. As shown in Fig. 3, expression of dynamin-K44A has no effect on agonist-induced Ras activation. In control experiments, Ras activation is markedly reduced after expression of activated Go12 subunits, which bind to and thereby activate Ras-GAP (37) (Fig. 3).

We next tested whether dynamin-K44A may interfere with the activation of Raf-1, MEK or MAP kinase. Fig. 4A shows that activation of Raf-1 by either EGF or TPA, as measured in an in vitro kinase reaction, is not affected by expression of dynamin-K44A. The same lysates were analyzed for the presence of activated MEK and MAP kinase using phospho-specific antibodies against the activated kinases. Fig. 4A shows that dynamin-K44A inhibits MAP kinase activation without affecting MEK activation.

In contrast, overexpression of WT-dynamin-1 does not abrogate MAP kinase activation, indicating that the inhibitory effect of dynamin-K44A is because of interference with endogenous dynamin activity, rather than to any nonspecific
Effect caused by dynamin overexpression (Fig. 4B). Instead, it is seen that overexpression of WT-dynamin-1 potentiates the activation of both endogenous and transfected MAP kinase (Fig. 4B), without affecting MEK activation (Fig. 4B, right panel). These results indicate that the activation of MAP kinase by MEK, rather than any upstream event, is regulated by dynamin.

**Dynamic-1 versus Dynamic-2**—Mammalian cells express three closely related dynamin isoforms: dynamin-1 is exclusively found in neuronal cells, dynamin-2 is ubiquitously expressed, whereas dynamin-3 is predominantly expressed in testis (38, 39). We compared the effects of dynamin-2 and dynamin-1 on the regulation of MAP kinase activation. As with dynamin-1-K44A, we find that dynamin-2-K44A inhibits MAP kinase activation but not MEK activation by EGF. In addition, overexpression of WT-dynamin-2 (like dynamin-1) potentiates MAP kinase activation in response to receptor stimulation (Fig. 4C). Thus, the actions of dynamin-1 and dynamin-2 in regulating the MAP kinase activation pathway are indistinguishable.

**Activated MEK Is Detected at the Plasma Membrane and in Intracellular Vesicles**—The above data strongly suggest that dynamin-regulated endocytosis of activated MEK from the plasma membrane is required for MAP kinase activation. Whereas activated MAP kinase can translocate to the nucleus, activated MEK remains in the cytoplasm (6, 7). We analyzed the subcellular distribution of endogenous activated MEK by immunofluorescence, using an antibody against phosphorylated MEK. In nonstimulated cells, only a faint, diffuse staining was observed. After stimulation with EGF or TPA, however, activated MEK is detected both at the plasma membrane and in intracellular vesicles (Fig. 5A). Although the endocytic nature of these vesicles remains to be confirmed, the results provide support for the notion that activated MEK is internalized from the plasma membrane following stimulation of cells with either receptor ligands or phorbol ester. Consistent with this, subcellular fractionation reveals that, whereas the total MEK pool is largely cytosolic, activated MEK is recovered in the particulate fraction consisting of membranes and cytoskeleton, but not in the cytosol (Fig. 5B). These experiments also reveal that only a relatively small fraction of total MEK is activated after agonist stimulation, presumably the pool that is localized to the plasma membrane.

**Conclusions**—A number of reports have suggested that activation of MAP kinase by receptor tyrosine kinases and G protein-coupled receptors requires their internalization. However, the mechanism whereby receptor endocytosis may contribute to MAP kinase activation has remained elusive to date, although the actions of dynamin-1 and dynamin-2 in regulating the MAP kinase activation pathway are indistinguishable.

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