p150Glued, dynein and microtubules are specifically required for activation of MKK3/6 and p38 MAPKs

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Running title: MKK3/6 directly interact with p150Glued dynactin
Summary

To look for regulators of the mitogen-activated protein kinase (MAPK) kinase 6 (M KK6), a yeast two-hybrid screen was initiated using MKK6 as bait. p150Glued dynactin, a key component of the cytoplasmic dynein/dynactin motor complex, was found to specifically interact with MKK6 and its close homologue MKK3. Silencing of p150Glued expression by small interference RNA reduced the stimulus-induced phosphorylation of MKK3/6 and p38 MAPKs. The similar adverse effect was also seen when the cytoplasmic dynein motor was disrupted by other means. Like p150Glued, MKK3/6 directly associate with microtubules. Disruption of microtubules prior to cell stimulation specifically inhibits the stimulus-induced phosphorylation of both MKK3/6 and p38 MAPKs. Our unexpected findings reveal a specific requirement for p150Glued/dynein/functional microtubules in activation of MKK3/6 and p38 MAPKs in vivo.
Introduction

MAPK-mediated intracellular signaling pathways play key roles in diverse cellular processes in eukaryotic organisms (1-3). The MAPK cascades transmit extracellular activating signals through sequential phosphorylation and activation of MKK kinase (MAP3K), MKK, and MAPK. At non-physiological concentrations in vitro, a purified MAP3K or MKK can directly bind and phosphorylate a particular MKK or MAPK respectively without a need for an additional regulatory molecule. However, it remains less clear in vivo how an activated upstream kinase finds and interacts with a specific inactive downstream kinase (among thousands of other cellular proteins). One can envision the involvement of either scaffold molecules or molecular motors that may bring MAP3K, MKK and MAPK to close physical proximity in cells. Indeed, several scaffolding molecules have been found which specifically group some or all components in a particular MAPK cascade to ensure signaling specificity and efficiency (4). In addition, certain MAPK components have also been found to bind cellular motors (5,6).

Cytoplasmic dynein is a microtubule-dependent minus-end moving motor. In complex and cooperation with the dynactin complex which includes p150\textsuperscript{Glued}, p50 dynamitin, Actin-related protein-1 (Arp-1) and several other polypeptides, dynein hydrolyzes ATP and drives movement of various cellular cargos especially those membranous vesicles (e.g., Golgi apparatus) (7,8).
To look for potential regulators of MKK6, an MKK acting specifically upstream of the p38 MAPKs (9), we carried out a yeast two-hybrid screen. Unexpectedly, p150Glued, a key component of the dynein/dynactin complex (7,8), was found to specifically interact with both MKK6 and MKK3 (a close homolog of MKK6 which also acts specifically upstream of the p38 MAPKs). Furthermore, we show that p150Glued, dynein, and functional microtubules are indispensable for activation of MKK3/6 and p38 MAPKs in vivo.

**Experimental Procedures**

**Cell Culture and reagents.** HeLa and HEK 293T cells were grown at 37°C with 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). Nocodazole was purchased from Calbiochem; Colchicine, Paclitaxel (Taxol), and erythro-9-[3-(2-hydroxynonyl)]adenine (EHNA) were from Sigma.

**Yeast two-hybrid screening.** The yeast strain AH109 (Mat a) expressing the bait protein GAL4-DBD-human MKK6(KM) was mated with Y187 (Mat α) containing the MATCHMAKER human skeletal muscle cDNA library following manufacturers instruction (BD Biosciences).

**Antibodies.** Anti-phospho-ERK1/2, anti-phospho-p38, and anti-phospho-MKK3/6
were from Cell Signaling Technology; anti-p150Glued from BD Biosciences; anti-β-tublin, anti-DIC, and anti-Flag from Sigma; anti-MKK3/6 from Upstate; anti-HA, anti-Xpress, anti-GFP, anti-His-tag, anti-Akt, and anti-ERK1 from Santa Cruz Biotechnology; anti-GST from Amersham Biosciences; and anti-giantin from Dr. HP. Hauri.

Cell lysis, kinase assays and Western blot

These were carried out as described in (10).

Microtubule preparation

Five grams of rat brain were homogenized in the PEM buffer (100 mM PIPES-NaOH, pH6.9, 1 mM EDTA, 1 mM MgSO₄, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin). Homogenates were centrifuged at 30,000 g for 15 minutes (min) at 4°C, and the supernatant was then clarified further by centrifugation at 180,000 g for 90 min at 4°C. The resulting supernatant was treated with 1 mM GTP and 20 µM Taxol at 37°C for 15 min to polymerize microtubules. The polymerized microtubules were pelleted by centrifugation at 30,000 g for 30 min. The pellets were washed once with the PEM buffer and resuspended in 250-400 µl of PEM buffer containing 1 mM GTP and 20 µM Taxol.

siRNA transfection
Human p150\textsuperscript{Glued} siRNA (sense: 5' uga ugg aac ugu uca agg c) was purchased from Dharmacon Inc. (Lafayette, CO). HeLa cells were transfected with siRNA at a final concentration of 100 nM per sample using LipofectAMINE 2000 (Invitrogen) following the manufacturer’s instruction.

**Results**

**p150\textsuperscript{Glued} specifically interacts with MKK3/6**

Using MKK6 as bait to screen a human skeletal muscle cDNA library, we found several clones displaying specific interaction with MKK6\textsuperscript{(KM)}, a kinase-dead form of MKK6, but not the control bait. Among them, there were two clones encoding different but overlapping regions of the carboxyl terminus of p150\textsuperscript{Glued} (Fig.1A). To further confirm the specific interaction between p150\textsuperscript{Glued} and MKK6, we studied their interaction in mammalian cells. 293T cells were transfected with either HA-MKK6 or HA-JNK2 (control). The endogenous p150\textsuperscript{Glued} was co-immunoprecipitated with HA-MKK6 but not HA-JNK2 (Fig.1B, lanes 2 and 6). Conversely, HA-MKK6 but not HA-JNK2 was co-immunoprecipitated with the endogenous p150\textsuperscript{Glued} dynactin (Fig.1B, lanes 3 and 7).

To delineate the MKK6-interacting regions on p150\textsuperscript{Glued}, clone 14 (Fig.1A) was
first divided into two fragments (i.e., amino acids (aa) 893-1025 and aa 1026-1292), each fused in-frame with Glutathione-S-transferase (GST). In a GST-pulldown assay, only the extreme carboxyl terminus of p150\textsuperscript{Glued} (i.e., aa 1026-1292) could bind MKK6 (our unpublished data). To fine map the MKK6-interacting domain, this 266 aa carboxyl terminus of p150\textsuperscript{Glued} was further divided into three smaller fragments (Fig.1C, left). Only CT3 (i.e., aa 1198-1292) could bind MKK6 in the GST-pulldown assays (Fig.1C, right).

To map the p150\textsuperscript{Glued}-interacting domain on MKK6, the amino terminus (NT: aa 1-59), and the kinase domain (KD: aa 60-257) of MKK6 were independently fused with the yeast Gal4 DNA binding domain (aa 1-147) and the fusion proteins were tested for their interaction with both clones 26 and 14 in yeast two-hybrid assays. Only the kinase domain of MKK6 displayed specific interaction with both clone 26 and 14 (Fig.1D).

As MKK3 is a close homologue of MKK6 with a nearly identical kinase domain, we tested whether MKK3 could interact with p150\textsuperscript{Glued}. Yeast vectors encoding either MKK3 or two other related stress-activated MKKs, JNKK1 (or MKK4) and JNKK2 (or MKK7) (1), were tested for their interaction with clones 26 and 14 in yeast two-hybrid assays. Indeed, only MKK3, but not JNKK1 and 2, specifically interacted with p150\textsuperscript{Glued} (Fig.1E).
M KK6 directly associates with tubulin

As p150 Glued is a microtubule-binding protein (8), we then tested whether MKK3/6 and p38 MAPK also associate with microtubules. Native microtubules were first polymerized and purified from rat brain (Fig. 2A) and the associated proteins were identified by Western blot. Like p150 Glued and dynein intermediate chain (DIC), a fraction of the endogenous MKK3/6 and p38 MAPK were also found to associate with microtubules (Fig. 2B). As a control, Akt was not present in our microtubule preparation (Fig. 2B). Using microtubules polymerized in vitro with the purified tubulins, we found that only the recombinant MKK6, but not the p38 MAPK or ATF2, co-sedimented with microtubules (Fig. 2C). This suggested that MKK6 but not the p38 MAPK directly binds to microtubules. To find out whether MKK6 also interacts with soluble tubulins, we carried out both in vivo and in vitro binding assays. In vivo, under conditions that tubulins did not polymerize, the endogenous MKK3/6 were specifically co-immunoprecipitated with the soluble tubulins as was p150 Glued dynactin (Fig. 2D). In vitro, when mixed with the purified tubulins, only the recombinant MKK6, but not the p38 MAPK or ATF2, interacted with tubulins in the GST-pulldown assays (Fig. 2E).

p150 Glued and dynein motor are involved in signal-induced phosphorylation of MKK3/6 and p38 MAPKs

To understand the cellular function of the interaction between MKK6 and
p150Glued, we first knocked down the expression of the endogenous p150Glued with siRNA before cells were stimulated by Sorbitol. We found that the Sorbitol-induced phosphorylation of both MKK3/6 and p38 MAPK were specifically reduced by the p150Glued-specific siRNA but not by a control siRNA (Fig.3A, lanes 5 and 6). Similarly, the p150Glued siRNA could also specifically reduce the tumor necrosis factor α (TNFα)-induced phosphorylation of MKK3/6 and p38 MAPK (our unpublished data). The decrease in the level of both phospho-MKK3/6 and phospho-p38 MAPK correlated with that of p150Glued expression (Fig.3A, lanes 3 and 6). Interestingly, the p150Glued siRNA had no obvious effect on the epidermal growth factor (EGF)-induced phosphorylation of ERKs, the prototypic members of the MAPK family (our unpublished data). As p150Glued is a key component of the dynein/dynactin complex, the reduction in p150Glued level would be expected to adversely affect the dynein motor function. To find out whether the dynein motor was involved, we first resorted to erythro-9-[3-(2-hydroxynonyl)]adenine (EHNA), a known inhibitor of the dynein ATPase activity (11,12). As a control, we first confirmed that EHNA caused dispersal of giantin in the Golgi apparatus (Our unpublished data), the aggregation of which being a known dynein-dependent process (13,14). Importantly, in cells pretreated with EHNA, the Sorbitol-induced phosphorylation of both MKK3/6 and p38 MAPKs was significantly
reduced in a dose-dependent manner (Fig.3B). In contrast, the EGF-induced phosphorylation of ERKs was not affected by similar EHNA treatment (Fig.3C). To further implicate a role for dynein in MKK3/6-p38 MAPK activation, we over-expressed p50 dynamitin, which is known to disrupt the dynein/dynactin complex and interferes with the dynein function (7,8). Indeed, the Sorbitol-induced phosphorylation of the endogenous p38 MAPK was reduced in cells over-expressing p50 dynamitin but not the empty vector (Fig.3D).

**Functional microtubules are required for signal-induced phosphorylation of MKK3/6 and p38 MAPKs**

As dynein is a microtubule-dependent motor and a fraction of MKK6, the p38 MAPK, and 150 Glued were all found to associate with microtubules (Fig.2), we next examined the involvement of microtubules. Before stimulating cells with Sorbitol, we first pretreated cells separately with Nocodazole, Colchicine, and Taxol, all being microtubule-interfering drugs which specifically disrupt the normal function of microtubules via different mechanisms (15). Indeed, the Sorbitol-induced phosphorylation of both MKK3/6 and the p38 MAPK were significantly reduced in cells pretreated with the drugs (Fig.4A). Similarly, the TNFα-induced phosphorylation of p38 MAPK could also be inhibited by these microtubule-interfering drugs (our unpublished data). Consistent with our previous data (Fig.3C), the EGF-induced ERK
phosphorylation and kinase activity were not affected by any of the three microtubule-interfering drugs (Fig. 4B).

Discussion

Associations between MAPK components and microtubules have long been observed. Both ERKs and JNKs were found to associate with microtubules in different cell types (16). However, the functional significance of such interactions remains poorly understood. Interestingly, links between MAPK components and microtubule-associated motors started to emerge in recent years. Mixed-lineage kinase 2 (MLK2), a member of the MAP3K superfamily, was found to interact with several members of the KIF3 kinesin motor complex (5). In addition, JNK-interacting proteins (JIPs), the scaffold proteins involved in organizing the JNK signaling complex (4), were found to be cargos for kinesin by interacting with the kinesin light chain (KLC) (6). In neuroblastoma cells, JIPs, together with other components of the JNK pathway, were transported to the neurite tips in a kinesin-dependent manner. In both cases above, however, it has not been addressed whether disruption of these interactions affects subsequent MAPK activation.

Similar to the role of KLC for kinesin motors, several components of the dynein/dynactin complex have been implicated in cargo-binding. For example, Arp-1 of the dynactin complex interacts with the Golgi-associated βIII spectrin (17). Dynein light
chain Tctex-1 interacts with the cytoplasmic portion of rhodopsin and the rhodopsin-containing vesicles can be moved in vitro on microtubules in a dynein-dependent manner (18). As to p150Glued, while its amino terminus binds microtubules, its carboxyl terminus is thought to participate in cargo-binding (7). Huntingtin-associated protein 1 (HAP1) was the first protein known to directly interact with the cargo-binding end of p150Glued and was recently confirmed to be a bona fide cargo of dynein (19-21). Interestingly, the binding site for HAP1 on p150Glued overlaps with that for MKK3/6. As p150Glued, dynein, and functional microtubules are all required for activation of MKK3/6 and the p38 MAPKs, we suggest that MKK3/6 may also serve as cargos for the dynein motor. In contrast to the p38 MAPKs, we find that the activation of ERKs by EGF is not dependent on p150Glued, dynein or functional microtubules. As ERKs can be activated by diverse stimuli in addition to EGF, it remains to be determined whether activation of ERKs under those conditions requires molecular motors and microtubules.

To our knowledge, the current study represents the first report of a functional link between MAPK components and dynein motors. Our data also suggest that the cellular motors can not only carry signaling complexes to different subcellular compartments but also play a more direct role in the process of signal transduction.
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**Legends to Figures**

**Fig. 1.** p150Glued interacts with MKK6 and MKK3. A, Two MKK6-interacting p150Glued clones (26 and 14) were schematically shown. The microtubule-binding site (black box) and two coiled-coil motifs (dotted box) of p150Glued were also indicated. N and C: the amino and carboxyl ends, respectively. The numbers denote the position of amino acids. B, HEK293T cells were transfected with either HA-MKK6 (left panel) or HA-JNK2 (right panel). Immunoprecipitation (IP) was performed using either anti-HA, anti-p150Glued, or a non-related antibody (IgG) followed by immunoblot (IB) with either anti-HA (top panel) or anti-p150Glued antibodies (bottom panel). WCE: whole cell extracts. C, The GST-fused p150Glued carboxyl fragments (schematically shown in the left panel) bound to the glutathione-Sepharose beads were incubated with equal amount of the recombinant His-MKK6. The MKK6 retained by GST-fusion proteins was revealed by immunoblotting (right panel). D and E, Different domains of MKK6, full-length MKK3, JNKK1, and JNKK2 were tested for their interaction with clones 26 and
14 in yeast two-hybrid assays. The results were summarized in D and E. The “+” and “−” signs denote growth and no growth on SD/-His/-Trp/-Leu/-Ade plates, respectively.

NT: amino terminus. KD: kinase domain. DBD: DNA-binding domain. AD: activation domain.

**Fig. 2.** MKK6 interacts with tubulin and microtubules. A, a Coomassie blue-stained gel showing our preparation of microtubules from rat brain. MT: microtubules; Sup: soluble supernatant. B, The endogenous proteins associated with microtubules (from A) were detected by immunoblot with various antibodies as indicated. C, The purified tubulins were first polymerized with Taxol and GTP and then incubated with various GST-fusion proteins. After centrifugation, the distribution of the fusion proteins in microtubule and supernatant fraction was analyzed by immunoblot. D, Soluble rat brain extracts were separately immunoprecipitated (IP) with the anti-β-tubulin and a non-related antibody (IgG) respectively and the bound proteins were detected by immunoblot. Input: 5% of extracts used for IP. E, Similar amount of GST-fusion proteins bound to the glutathione-Sepharose beads were separately incubated with equal amount of the purified tubulin. The bound tubulin was detected by immunoblot. Input: 5% of GST-fusion proteins used in the pulldown assays.

**Fig. 3.** p150Glued and the dynein motor are required for activation of MKK3/6 and the p38 MAPKs. A, HeLa cells were transfected with either a control or p150Glued-specific
siRNA. 20 and 48 hours (hr) after siRNA transfection, cells were either left untreated (NT) or treated with Sorbitol (Sorb, 0.4 M) for 20 min before harvest. WCE were then subjected to immunoblot analysis. B, HeLa cells were either pretreated with vehicle (water, lanes 1, 2, 6) or increasing amount of EHNA for either 30 min (lanes 3-5) or 1 hr (lanes 7-10) before Sorbitol stimulation. WCE were then analyzed by immunoblot. NT: untreated. C, Same as in B except that the serum-starved (for 12 hrs) HeLa cells were stimulated with EGF (100 ng/ml, 10 min). SF: serum-free. D, 293T cells were transfected with vectors encoding either GFP or GFP-p50 dynamitin. 24 hr after transfection, the cells were stimulated with 0.2 M Sorbitol for 20 min before harvest. WCE were analyzed by immunoblot.

Fig. 4. Functional microtubules are required for activation of MKK3/6 and the p38 MAPKs. A, B, HeLa cells were either left untreated (NT) or pretreated with nocodazole (Noc, 1 μg/ml, 4 hr), paclitaxel (Tax, 1 μM, 6 hr) or colchicine (Col, 0.4 μM, 8 hr) before stimulation with either Sorbitol (A, 0.4 M for 20 min) or EGF (B, 100 ng/ml for 10 min). WCE were analyzed by immunoblot. The activity of the endogenous ERKs was also directly measured in the immune-complex kinase assays (KA) using myelin basic protein (MBP) as a substrate.
Fig. 1 Cheung & Wu
Fig. 2. Cheung & Wu
Fig. 3. Cheung & Wu
Fig. 4. Cheung & Wu
p150\textsuperscript{Glued}, dynein and microtubules are specifically required for activation of MKK3/6 and p38 MAPKs

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