Activation of p38 Mitogen-activated Protein Kinase by PYK2/Related Adhesion Focal Tyrosine Kinase-dependent Mechanism*

(Received for publication, November 23, 1998, and in revised form, February 5, 1999)

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The stress-activated p38 mitogen-activated protein kinase (p38 MAPK), a member of the subgroup of mammalian kinases, appears to play an important role in regulating inflammatory responses, including cytokine secretion and apoptosis. The upstream mediators that link extracellular signals with the p38 MAPK signaling pathway are currently unknown. Here we demonstrate that p125 focal adhesion kinase-related tyrosine kinase RAFTK (also known as PYK2, CADTK) is activated specifically by methylmethane sulfonate (MMS) and hyperosmolarity but not by ultraviolet radiation, ionizing radiation, or cis-platinum. Overexpression of RAFTK leads to the activation of p38 MAPK. Furthermore, overexpression of a dominant-negative mutant of RAFTK (RAFTK K-M) inhibits MMS-induced p38 MAPK activation. MKK3 and MKK6 are known potential constituents of p38 MAPK signaling pathway, whereas SEK1 and MEK1 are upstream activators of SAPK/JNK and ERK pathways, respectively. We observe that the dominant-negative mutant of MKK3 but not of MKK6, SEK1, or MEK1 inhibits RAFTK-induced p38 MAPK activity. Furthermore, the results demonstrate that treatment of cells with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, tetra(acetoxyethyl)-ester, a membrane-permeable calcium chelator, inhibits MMS-induced activation of RAFTK and p38 MAPK. Taken together, these findings indicate that RAFTK represents a stress-sensitive mediator of the p38 MAPK signaling pathway in response to certain cytotoxic agents.

The mitogen-activated protein kinases (MAPKs)† are induced in response to diverse classes of inducers in the transduction of signals from the cell membrane to the nucleus. MAPKs are proline-directed Ser/Thr protein kinases that are regulated by extracellular signals including growth factors and cellular stress (1–3). The well characterized MAPK subfamily includes ERK1 and ERK2, which are activated by growth factors via the conserved Ras/Raf/MEK pathway (4–7). c-Jun N-terminal protein kinases (JNKs) or stress-activated protein kinases (SAPKs) represent a second class of the mammalian MAPKs, which are primarily activated in response to tumor necrosis factor, interleukin-1, UV-, and DNA-damaging agents (5, 8–11). A recently identified novel protein tyrosine kinase, related adhesion focal tyrosine kinase (RAFTK) (12) (also known as Pyk2, Refs. 13 and 14); CADTK, Ref. 15) has been shown to be involved upstream to ERKs and JNK signaling pathways (14, 16). RAFTK is also a close relative to pp125 FAK tyrosine kinase and is activated by various extracellular signals that increase intracellular calcium concentrations (13). Moreover, RAFTK can tyrosine phosphorylate and modulate the action of ion channels and appears to function as an intermediate that links various calcium signals with both short- and long-term responses in neuronal cells (13).

An additional class, which presents substantial similarity to the Saccharomyces cerevisiae HOG1 kinase involved in response to increased extracellular osmolarity (17), is p38 MAPK. p38 MAPK can also be activated by changes in osmolarity, lipopolysaccharides, and in response to DNA-damaging agents (3, 18–20). Other studies have demonstrated that the Rho GTPases and multiple p21 activated kinases regulate p38 MAPK (21, 22). Moreover, in contrast to activation of ERKs, interleukin-1 and tumor necrosis factor are potent activators of p38 MAPK, suggesting upstream signals via Ras does not play a key role in p38 MAPK activation. Previous studies have shown that diverse genotoxic agents activate p38 MAPK and that this response is mediated by c-Abl protein tyrosine kinase-dependent and -independent mechanisms (20). Taken together, although certain insights are available, the precise upstream mechanisms responsible for activation of p38 MAPK are presently unclear.

The results of the present study demonstrate that in contrast to ionizing radiation (IR), cis-platinum (CDDP), or ultraviolet radiation (UV), RAFTK is activated in response to certain cytotoxic agents such as methylmethane sulfonate (MMS) or hyperosmolarity. Overexpression of RAFTK leads to the activation of p38 MAPK. Furthermore, the dominant-negative mutant of RAFTK inhibits p38 MAPK activation in response to certain genotoxic agents. These findings indicate that RAFTK represents a stress-sensitive mediator of the p38 MAPK signaling pathway in response to certain cytotoxic agents.
tand of MKK3 but not of MKK6 (potential upstream mediators of p38 MAPK signaling pathway) inhibits RAFTK-induced p38 MAPK activity. These findings indicate that RAFTK represents a stress-sensitive mediator of the p38 MAPK pathway.

**MATERIALS AND METHODS**

**Cell Culture and Reagents**—Human U-937 myeloid leukemia cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. PC12 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum and antibiotics. 293T cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics. Cells (1 × 10⁶/100-mm culture dish) were plated 24 h before treating with 1 mM MMS (Sigma), 100 μM CDDP (Sigma), 500 mM NaCl, 20 μM BAPTA-AM (Sigma), or 20 Gy IR at room temperature with a Gammacell 1000 (Atomic Energy of Canada, Ottawa) under aerobic conditions with a 137Cs source emitting at a fixed dose rate of 0.76 Gy min⁻¹ as determined by dosimetry. Cells were also treated with 40 J/m² UV (UV StratalinkerTM, 1800, Stratagene).

**Immunoprecipitation and Immunoblot Analysis**—U-937 or PC12 cells were treated with 1 mM MMS, 500 mM NaCl, 100 μM CDDP, 20 Gy IR, 20 μM BAPTA-AM, or 40 J/m² UV and harvested at different time intervals. The cells were subjected to immunoprecipitation with anti-RAFTK antibody as described (23) and analyzed by immunoblotting with anti-P-Tyr. U-937 cells were also treated with 1 mM MMS and harvested at different time intervals. Total cell lysates were subjected to immunoblotting with anti-phospho-MKK3/MKK6 antibody (New England Biolabs). The antigen-antibody complexes were visualized by chemiluminescence (ECL, Amersham).

**Transient Transfections, Immunoprecipitations, and Immune Complex Kinase Assays**—293T cells were transiently transfected with vector or Flag-RAFTK with HA-p38 MAPK or pEBG-SAPK using a standard calcium phosphate method as described by Kharbanda et al. (24). Cells were co-transfected separately with dominant-negative mutants of Flag-MKK3, Flag-MKK6, MEK1, or SEK1 with HA-p38 MAPK. After 48 h of transfections, cells were washed with phosphate-buffered saline and lysed in 1 mL of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 10 μM leupeptin and aprotinin) as described by Kharbanda et al. (25). Total cell lysates were subjected to immunoprecipitation with anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4 °C and then for 1 h after addition of protein A-Sepharose. The immune complexes were washed three times with lysis buffer, once with kinase buffer, and resuspended in kinase buffer containing [γ-32P]ATP (6,000 Ci/mmol; NEN Life Science Products) and GST-ATF2 (1–102) as substrate (26). The reactions were incubated for 15 min at 30 °C and terminated by the addition of SDS sample buffer. The proteins were analyzed by 10% SDS-PAGE and autoradiography. The immune complexes were also analyzed by immunoblotting with anti-HA, anti-JNK (Santa Cruz Biotechnology, anti-RAFTK (12), or anti-P-Tyr (4G10; Upstate Biotechnology, Lake Placid, NY).

PC12 cells were transiently transfected with vector or Flag-RAFTK (K-M) with HA-p38 MAPK using LipofectAMINETM (Life Technologies, Inc.). After transfection, cells were treated with MMS, and total cell lysates were subjected to incubation with anti-HA antibody. The immune complexes were analyzed by immunoprecipitation with anti-HA antibody (Santa Cruz Biotechnology) and anti-HA protein precipitates for GST-ATF2 phospho-RAFTK as described. The reactions were incubated for 15 min at 30 °C and terminated by the addition of SDS sample buffer. The proteins were analyzed by 10% SDS-PAGE and autoradiography. The immune complexes were also analyzed by immunoblotting with anti-HA.

**RESULTS AND DISCUSSION**

Previous studies have demonstrated that certain agents, such as phorbol esters and sorbitol, activate RAFTK (15, 16). Activation of RAFTK by tumor necrosis factor or UV, as detected by its phosphorylation on tyrosine, is controversial and may be cell type specific (15, 16). RAFTK has also been linked to cell and inducer type specific activation of JNK/SAPK and ERK1/2 signaling pathways (14–16).

To determine whether RAFTK is involved in activation of p38 MAPK pathway, we transiently overexpressed wild type RAFTK along with HA-tag p38 MAPK in 293T cells and analyzed the anti-HA protein precipitates for GST-ATF2 phosphorylation. In a parallel experiment, we also overexpressed wild type RAFTK with pEBG-SAPK, and GST protein precipitates were analyzed for GST-Jun phosphorylation. The results demonstrate increased GST-ATF2 phosphorylation (increased p38 MAPK activity) in cells transfected with wild type RAFTK compared with that with vector (Fig. 1A). The activation of p38 MAPK was dependent on expression of the RAFTK protein (data not shown). Similar results were obtained when wild type RAFTK transfected cells were analyzed for JNK activity (Fig. 1B). The RAFTK-induced increase in p38 MAPK or JNK activities occurred in the absence of changes in p38 or JNK protein levels, respectively (Fig. 1, A and B). To confirm expression of RAFTK, we assayed the anti-RAFTK immune complexes for reactivity with anti-RAFTK or anti-P-Tyr antibody. The results demonstrate increased protein levels and tyrosine phosphorylation of RAFTK in overexpressed cells (Fig. 1, A and B). Taken together, these findings indicate that RAFTK acts as an upstream mediator of the p38 MAPK pathway.

Because RAFTK is activated in part by phosphorylation on
Tyr (13), we next examined the effect of different potent p38 MAPK activators on the status of RAFTK phosphorylation in human myelomonocytic leukemia (U-937) cells and in rat pheochromocytoma (PC12) cells. In contrast to IR or CDDP, stimulation with MMS or hyperosmolarity lead to enhance tyrosine phosphorylation of RAFTK in U-937 and PC12 cells (Fig. 2). Exposure of U-937 or PC12 cells to UV caused little, if any, increase in the tyrosine phosphorylation of RAFTK (Fig. 2A and data not shown). Moreover, all of these stress inducers activate p38 MAPK in both cell types (Fig. 2, B and D and data not shown). Taken together, these findings indicated that not all stress signals stimulate activation of RAFTK.

Our findings suggest that RAFTK acts as an upstream mediator of the p38 MAPK pathway. To further confirm a direct role for RAFTK in MMS-induced activation of p38 MAPK, PC12 cells were transiently transfected with a dominant-negative mutant of RAFTK (RAFTK K-M). The cells were also cotransfected with HA-p38 MAPK. PC12 cells were used in this study because RAFTK is expressed in these cells and the transfection efficiency in PC12 cells is comparatively better than that in U-937 cells. After transfection, cells were treated with MMS in the absence of 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, tetra(acetoxymethyl)-ester (BAPTA-AM). BAPTA-AM is a membrane-permeable calcium chelator and has been shown to inhibit JNK activation in response to various inducers that raise intracellular calcium, such as angiotensin II, thapsigargin, or ionophore (27). Other studies have shown that BAPTA-AM acts also as an intracellular calcium channel blocker (28). The results demonstrate that MMS-induced tyrosine phosphorylation of RAFTK was significantly inhibited in cells that were treated in the presence of BAPTA-AM (Fig. 4A). Moreover, in the presence of BAPTA-AM, p38 MAPK activity induced in response to MMS is also significantly inhibited but not completely blocked (Fig. 4B, top panel). Furthermore, the inhibition of MMS-induced p38 MAPK activity by BAPTA-AM was without any significant effect on p38 MAPK protein levels (Fig. 4B, bottom panel). Treatment of U-937 cells with MMS in the presence and absence of BAPTA-AM. These results suggest that MMS-induced activation of RAFTK is mediated, at least in part, by the increase in intracellular calcium levels that is induced by this stimulus. Thus inhibition in MMS-induced p38 MAPK activity by BAPTA-AM further confirms the role of RAFTK in mediating p38 MAPK activation in response to MMS. Because MMS-induced activation of p38 MAPK is not completely blocked in the presence of BAPTA-AM, it is likely that MMS activates the p38 MAPK by multiple upstream pathways. Therefore, RAFTK acts as an upstream activator of the p38 MAPK signaling pathway at least in the cellular response to MMS.

RAFTK is activated by various extracellular signals that increase intracellular calcium levels such as carbamoyl, angiotensin II, or sorbitol (13, 16). To examine the role of calcium on MMS-induced activation of RAFTK and induction of p38 MAPK activity, we treated U-937 cells with MMS in the presence or absence of 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, tetra(acetoxymethyl)-ester (BAPTA-AM). BAPTA-AM is a membrane-permeable calcium chelator and has been shown to inhibit JNK activation in response to various inducers that raise intracellular calcium, such as angiotensin II, thapsigargin, or ionophore (27). Other studies have shown that BAPTA-AM acts also as an intracellular calcium channel blocker (28). The results demonstrate that MMS-induced tyrosine phosphorylation of RAFTK was significantly inhibited in cells that were treated in the presence of BAPTA-AM (Fig. 4A). Moreover, in the presence of BAPTA-AM, p38 MAPK activity induced in response to MMS is also significantly inhibited but not completely blocked (Fig. 4B, top panel). Furthermore, the inhibition of MMS-induced p38 MAPK activity by BAPTA-AM was without any significant effect on p38 MAPK protein levels (Fig. 4B, bottom panel). Treatment of U-937 cells with MMS in the presence and absence of BAPTA-AM. These results suggest that MMS-induced activation of RAFTK is mediated, at least in part, by the increase in intracellular calcium levels that is induced by this stimulus. Thus inhibition in MMS-induced p38 MAPK activity by BAPTA-AM further confirms the role of RAFTK in mediating p38 MAPK activation in response to MMS. Because MMS-induced activation of p38 MAPK is not completely blocked in the presence of BAPTA-AM, it is likely that MMS activates the p38 MAPK by multiple upstream pathways. Therefore, RAFTK.

![Fig. 2. Phosphorylation of RAFTK and activation of p38 MAPK by diverse cytotoxic agents in U-937 and PC12 cells. A, U-937 cells were treated with 1 mM MMS, 500 mM NaCl, 100 μM CDDP, or 40 J/m2 UV and harvested at the indicated times. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK antibodies and analyzed by immunoblotting with anti-P-Tyr. B, U-937 cells were treated with 1 mM MMS for 2 h, 500 mM NaCl for 30 min, 40 J/m2 UV for 15 min, 20 Gy IR for 4 h, or 100 μM CDDP for 3 h. Total cell lysates were also subjected to immunoprecipitation with anti-p38 MAPK antibodies, and in vitro immune complex kinase assays were performed using GST-ATF2 as substrate. PC12 cells were treated with 1 mM MMS for 2 h or 500 mM NaCl in 15 min. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK (C) or anti-p38 (D) and analyzed by immunoblotting with anti-P-Tyr (C) or in vitro immune complex kinase assays (D) as described.

![Fig. 3. PC12 cells were transiently transfected with vector or RAFTK K-M. The cells were also co-transfected with HA-p38 MAPK. 48 h after transfection, cells were treated with 1 mM MMS and harvested after 1 h. Cell lysates were subjected to immunoprecipitation with anti-HA antibody, and the immunoprecipitates were analyzed by in vitro immune complex kinase assays using GST-ATF2 as a substrate (top panel). Anti-HA immunoprecipitates were analyzed by immunoblotting with anti-RAFTK (second panel). Total lysates were also analyzed by immunoblotting with anti-RAFTK antibodies, and data not shown). Moreover, all of these stress inducers activate p38 MAPK in both cell types (Fig. 2, B and D and data not shown). Taken together, these findings indicated that not all stress signals stimulate activation of RAFTK.

Our findings suggest that RAFTK acts as an upstream mediator of the p38 MAPK pathway. To further confirm a direct role for RAFTK in MMS-induced activation of p38 MAPK, PC12 cells were transiently transfected with a dominant-negative mutant of RAFTK (RAFTK K-M). The cells were also cotransfected with HA-p38 MAPK. PC12 cells were used in this study because RAFTK is expressed in these cells and the transfection efficiency in PC12 cells is comparatively better than that in U-937 cells. After transfection, cells were treated with MMS in the absence of 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, tetra(acetoxymethyl)-ester (BAPTA-AM). BAPTA-AM is a membrane-permeable calcium chelator and has been shown to inhibit JNK activation in response to various inducers that raise intracellular calcium, such as angiotensin II, thapsigargin, or ionophore (27). Other studies have shown that BAPTA-AM acts also as an intracellular calcium channel blocker (28). The results demonstrate that MMS-induced tyrosine phosphorylation of RAFTK was significantly inhibited in cells that were treated in the presence of BAPTA-AM (Fig. 4A). Moreover, in the presence of BAPTA-AM, p38 MAPK activity induced in response to MMS is also significantly inhibited but not completely blocked (Fig. 4B, top panel). Furthermore, the inhibition of MMS-induced p38 MAPK activity by BAPTA-AM was without any significant effect on p38 MAPK protein levels (Fig. 4B, bottom panel).
may function as one and not the only upstream mediator in the MMS response.

The p38 MAPK signal transduction pathway is activated by diverse classes of stimuli such as proinflammatory cytokines, environmental stress, and DNA damage (1, 20, 29). These stimuli can also activate other signal transduction pathways such as ERKs or JNKs (5, 8–10, 30). Moreover, the role of selective upstream activators of these pathways such as MKK3 and MKK6 for p38 MAPK, MEK1 and MEK2 for ERK, and MKK4 (SEK1) and MKK7 are necessary for JNK activation (29, 31–34). To determine whether RAFTK-induced activation of p38 MAPK involves MKK3 or MKK6, we used catalytically inactive mutants of MKK3 (MKK3 K-A) and MKK6 (MKK6 K-A) (31), which act as dominant-negative inhibitors. 293T cells were transiently transfected with wild-type RAFTK, Flag-MKK3 K-A or Flag-MKK6 K-A. After 48 h, total cell lysates were immunoprecipitated with anti-HA antibody and in vitro immune complex kinase assays using GST-ATF2 as substrate (top panel). Total cell lysates were also analyzed by immunoblotting with anti-p38 MAPK antibody (bottom panel). The p38 MAPK signal transduction pathway is activated by diverse classes of stimuli such as proinflammatory cytokines, environmental stress, and DNA damage (1, 20, 29). These stimuli can also activate other signal transduction pathways such as ERKs or JNKs (5, 8–10, 30). Moreover, the role of selective upstream activators of these pathways such as MKK3 and MKK6 for p38 MAPK, MEK1 and MEK2 for ERK, and MKK4 (SEK1) and MKK7 are necessary for JNK activation (29, 31–34). To determine whether RAFTK-induced activation of p38 MAPK involves MKK3 or MKK6, we used catalytically inactive mutants of MKK3 (MKK3 K-A) and MKK6 (MKK6 K-A) (31), which act as dominant-negative inhibitors. 293T cells were transiently transfected with wild-type RAFTK, Flag-MKK3 K-A or Flag-MKK6 K-A with HA-p38 MAPK. After 48 h, anti-HA immunoprecipitates were analyzed for p38 MAPK activity. The results demonstrate that RAFTK-induced activation of p38 MAPK is significantly inhibited by MKK3 K-A but not by MKK6 K-A (Fig. 5A). As a control, 293T cells were also transfected separately with dominant-negative mutants of SEK1 or MEK1 in the presence of wild-type RAFTK and HA-p38 MAPK and anti-HA immunoprecipitates were analyzed for p38 MAPK activity. The results demonstrate that in contrast to MKK3 K-M, dominant-negative mutants of SEK1 or MEK1 are not associated with significant inhibition in p38 MAPK activity (Fig. 5B). To assess whether treatment of cells with MMS induces phosphorylation of MKK3, U-937 cells were treated with MMS and harvested at different time intervals. Cell lysates were then analyzed by immunoblotting with antibody raised against the phosphorylated form of MKK3. The results demonstrate that treatment with MMS is associated with induction in the phosphorylated form of MKK3 (Fig. 5C). Taken together, these findings demonstrated that RAFTK acts upstream to MKK3 in the stress response to MMS.

The present results demonstrate that diverse types of stress inducers induce p38 MAPK activity in U-937 and PC12 cells. The findings also demonstrate that MMS and NaCl induce p38 MAPK activity by RAFTK-dependent mechanisms. Previous work has shown that IR- and CDDP-induced activation of p38 MAPK occurs by c-Abl tyrosine kinase-dependent mechanisms. Furthermore, MMS, UV, tumor necrosis factor, and NaCl induce p38 MAPK by c-Abl-independent mechanisms. The find-
ing correlates that MMS- and NaCl-induced p38 MAPK activity requires activation of RAFTK, further supports distinct signaling events used by various cytotoxic agents. The UV response in mammalian cells is initiated in an extranuclear compartment (35). The finding that Ha-Ras contributes to the induction of JNK by UV is also consistent with the hypothesis that UV response is initiated in the cytoplasm. MMS is a monofunctional alkylating agent that alkylates DNA and damages membrane proteins (36, 37). Therefore, MMS may also activate p38 MAPK activity by DNA damage-independent mechanisms. In this context, taken together with our previous findings (20) in contrast to DNA-damaging agents, MMS and NaCl induces p38 MAPK activity by RAFTK-dependent and c-Abl-independent mechanisms. Therefore, these results demonstrate that activation of the stress response to diverse agents can be distinguished by c-Abl-dependent, RAFTK-dependent, and/or other tyrosine kinase-dependent mechanisms.

Acknowledgments—We thank Drs. John Kyriakis, Joseph Avruch, and Leonard Zon for providing various SAPK cDNAs and anti-GST-SAPK antibody. We thank Rebecca Farber for technical assistance.

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