Activated Ki-Ras Suppresses 12-O-Tetradecanoylphorbol-13-acetate-induced Activation of the c-Jun NH₂-Terminal Kinase Pathway in Human Colon Cancer Cells

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ABSTRACT

Although the frequency of activated Ki-ras genes is high in human colorectal tumors, much less is known of activated Ki-ras-mediated signaling pathways. Using gene targeting, we examined HCT116 cells that contain the Gly-13→Asp mutation of Ki-ras and activated Ki-ras-disrupted clones derived from HCT116. 12-O-Tetradecanoylphorbol-13-acetate (TPA) induced immediate early genes, such as c-Jun, c-Fos, and Egr-1 in activated Ki-ras-disrupted clones, whereas c-Jun induction was rare in HCT116. TPA induced both phosphorylation of stress-activated protein kinase kinase 1 (SEK1) and c-Jun NH₂-terminal kinase (JNK) in the activated Ki-ras-disrupted clones but not in HCT116. On the other hand, TPA-induced mitogen-activated protein kinase kinase 1/2 (MEK1/2)-extracellular signal-regulated kinase (ERK) activation was equally induced between HCT116 and the Ki-ras-disrupted clones. Furthermore, TPA-induced SEK1-JNK activation was observed in a DLD-1-derived activated Ki-ras-disrupted clone but not in DLD-1. The TPA-induced SEK1-JNK activation in these disrupted clones was completely inhibited by the protein kinase C (PKC) inhibitor, GF109203X (1 μM), but not by another PKC inhibitor, H7 (50 μM), whereas TPA-induced MEK1/2-ERK activation was partially and completely inhibited by GF109203X (1 μM) and H7 (50 μM), respectively. A phosphoinositol 3-kinase inhibitor, LY294002, did not inhibit the TPA-induced SEK1-JNK activation. Taken together, these results suggest that activated Ki-Ras-mediated signals are involved in the SEK1-JNK pathway through a PKC isotype that is distinct from that involved in MEK1/2-ERK activation in human colon cancer cells and independent of phosphoinositol 3-kinase activation, and the imbalance between ERK and JNK activity caused by activated Ki-Ras may play critical roles in human colorectal tumorigenesis.

INTRODUCTION

Ras has been implicated in controlling cell proliferation, differentiation, and apoptosis. The ras family consists of three known functional genes, Ha-ras, Ki-ras, and N-ras, which encode highly similar proteins, all with a molecular mass of 21 kDa (1). The activated ras oncogenes can transform mammalian cells in culture and have been implicated in the formation of a high proportion of human tumors (1, 2). Activated Ki-ras oncogenes were found in the majority of exocrine pancreas carcinomas (3) and in a high frequency of colorectal tumors (4, 5), whereas the N-ras gene was frequently mutated in myeloid leukemia, indicating a possible correlation between tumor type and ras gene mutation (2).

Although activated Ki-ras-mediated signaling pathways largely remain unknown, the Ha-ras-mediated signaling pathway has been extensively investigated, especially with regard to MAPKs, which are classified in three subfamilies: the ERK, JNK/SAPK, and p38 kinase subfamilies (6, 7). Oncogenic Ha-ras activates p42/ERK2 in Swiss 3T3 cells without requiring growth factors, and this activation of ERK2 is constitutive and has two components, one of which depends on PKC whereas the other is independent of PKC (8). In Rat-1 cells, p21-Ha-ras mediates platelet-derived growth factor-induced activation of ERK2 but not phorbol ester TPA-induced activation of ERK2 (9), whereas the dominant inhibitory mutant of Ha-ras, Ha-ras (Asn-71), antagonized the growth factor- and TPA-induced activation of p44/ERK1 and ERK2 in PC12 cells (10, 11). Activated Ha-ras also causes a marked increase in the transcriptional activity of c-Jun through phosphorylation of c-Jun on Ser-63 and Ser-73 (12, 13), which is phosphorylated by M, 46,000 and 55,000 JNKs termed JNK1 and JNK2, respectively (14). Both ERK and JNK phosphorylate transcriptional factors (15). ERK1 and ERK2 phosphorylate and potentiate the activity of TCF/Elk-1 and thereby induce c-fos (16, 17), whereas JNK phosphorylates and potentiates the activity of c-Jun and ATF2 (14, 18, 19). Ha-Ras activates two protein kinases, Raf-1 and MEKK (20). Raf-1 contributes directly to ERK activation but not to JNK activation, whereas MEKK participates in JNK activation. Hence, there seem to be two distinct Ha-Ras-dependent MAPK cascades (20).

To elucidate how activated Ki-Ras is involved in human colorectal tumorigenesis, we earlier established HCT116- and DLD-1-derived clones, in which activated Ki-ras was disrupted through gene targeting (21). Using these cells, we found that activated Ki-Ras is involved in the deregulation of c-myc (21) and in up-regulation of vascular endothelial growth factor (22, 23), suggesting that activated Ki-Ras is critically involved in tumorigenicity. We also reported that activated Ki-Ras enhanced the sensitivity of ceramide-induced apoptosis, without JNK/SAPK or ERK activation in HCT116 (24) and was involved in the suppression of c-jun expression induced by serum stimulation (25).

To determine how activated Ki-Ras is involved in the MAPK pathway in human colon cancer cells, we analyzed HCT116, DLD-1, and the activated Ki-ras-disrupted clones derived from HCT116 and DLD-1. We obtained evidence that activated Ki-Ras suppresses SEK1-JNK activation induced by TPA in human colon cancer cells, the mechanisms of which are dependent on a PKC isotype that is distinct from that involved in MEK1/2-ERK activation but not in PI3K activation.

MATERIALS AND METHODS

Cells and Cell Culture. The human colon cancer cell lines HCT116 (ATCC CCL247) and DLD-1 (ATCC CCL241) both have one normal Ki-ras allele and one Ki-ras allele with a point mutation at codon 13 that converts Gly to Asp. HKc3, HK2–8, HKh2, HK2–6, and DK0–4 are Ki-ras-disrupted clones from HCT116 and DLD-1, respectively (21). Cells were grown in DMEM supplemented with 10% FCS in a humidified atmosphere of 10% CO₂.

Chemicals. 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7; Ref. 26), purchased from Seikagaku Kogyo (Tokyo, Japan), was used at the concentration of 50 μM. GF109203X was from Sigma Chemical Co. (St. Louis, MO). LY294002, TPA, and anisomycin were from Calbiochem (La Jolla, CA).

Serum Starvation and Stimulation. Cells were washed twice with PBS and cultured in serum-free DMEM for 24 h, followed by the addition of 10% FCS or TPA (50 ng/ml). PKC inhibitors were preincubated for 1 h before TPA stimulation. Cells were also stimulated by UV (10,000 μJ/cm²), H₂O₂ (1 mM), and anisomycin (50 μg/ml). Cell extracts were prepared at 1 h after each treatment.
**Northern Blot Analysis.** Total cellular RNA (15 mg) was separated by electrophoresis in a 1% agarose-formaldehyde gel, transferred to nylon membrane (Hybond N+; Amersham, Buckinghamshire, England), and hybridized with a probe for c-jun, as described (21). The washed filters were exposed to Kodak XAR-5 film for 2 days.

**Antibodies.** Antibodies used for Western blotting were as follows: anti-ERK1 (sc-44), JNK1 (sc-571), c-Fos (sc-52), Egr-1 (sc-110), and ATF2 (sc-187) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-p38, MEK1/2, and CREB were from New England Biolabs (Beverly, MA); anti-c-Jun was from Transduction Laboratories (Lexington, KY).

**Phosphospecific Antibodies and Assays of JNK, SEK1, ERK, MEK1/2, and c-Jun Activity.** Phospho-specific antibodies detected for activation of kinases or transcriptional factors were purchased from New England Biolabs: phosphospecific MAPK antibody detecting phosphorylated ERK1 and ERK2 on Tyr-204; anti-MEK1/2 on Ser-217/Ser-221; anti-SAPK/JNK on Thr-183/Tyr-185; anti-p38 MAPK on Thr-180/Tyr-182; anti-SEK1/MKK4 on Thr-223; anti-c-Jun on Ser-63; and anti-CREB on Ser-133. Activity of JNK, SEK1, ERK1, MEK1/2, and c-Jun were determined by using these phosphospecific antibodies (13, 27–29).

**Western Blot Analysis.** Proteins were extracted using 1× SDS sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 2% β-mercapto-ethanol, and 1 mM orthovanadate, at serum starvation and at the indicated time points after treatment with FCS, TPA, UV, H2O2, anisomycin, or DiC8. Equal amounts of total lysate were electrophoresed in 8% or 10% SDS-polyacrylamide gels at a constant current of 25 mA for 90 min, followed by transfer to a polyvinylidene fluoride membrane (Micron Separations, Inc., Westborough, MA). Western blotting was performed using the ECL system (Amersham), with the antibodies described above. Each blot was performed at least five times using cell lysates extracted at different times, with identical results.

**RESULTS**

**Suppression of TPA-induced c-Jun Expression and c-Jun Phosphorylation at Ser-63 in Human Colon Cancer Cells with Activated Ki-ras.** We previously established HCT116-derived clones HKe3, HKh2, and HK2–8, in which activated Ki-ras was disrupted through gene targeting (21). c-jun mRNA expression was inhibited by serum in HCT116 with activated Ki-ras expression in HCT116 with activated Ki-ras. We disrupted Ki-ras by TPA stimulation (25). To simplify this serum-induced biological phenomenon, TPA was used for stimulation. HCT116 and HKe3 cells were serum starved for 24 h and then stimulated by TPA. In HKe3, c-jun induction was clearly observed at 6 h after TPA stimulation, and expression was most strongly observed at 1 h after stimulation (Fig. 1A). Conversely, c-Jun was rarely observed during the 6 h after TPA stimulation in HCT116 (Fig. 1A). Taken together, these results suggested that activated Ki-Ras-mediated signals suppress growth factor-induced c-jun expression in human colon cancer cells. To determine how the activated Ki-Ras is involved in suppression of c-jun induction, expression of c-Jun, induced by TPA, was examined using HCT116 and HKe3. c-Jun was not evident under exponential growth or serum starvation conditions in either line (Fig. 1B). c-jun expression induced by serum in HCT116 was lower than that in HKe3, and this suppression was more evident when cells were stimulated by TPA (Fig. 1B). The transcriptional activity of c-jun is regulated by phosphorylation at Ser-63 and Ser-73 (13). To determine whether the induced c-jun is transcriptionally activated, immunobLOTS were prepared using antiphosphospecific c-jun (Ser-63) antibody. c-Jun was phosphorylated at Ser-63 in HKe3 in case of stimulation with serum or TPA, whereas c-jun on Ser-63 was rarely phosphorylated in HCT116 with TPA (Fig. 1B). All these findings suggest that loss of functional activated Ki-Ras leads to restoration of TPA-induced c-Jun expression and that phosphorylation of the critical site for activation of c-Jun in HKe3.

We then asked whether suppression of the TPA-induced c-jun expression in HCT116 with activated Ki-ras was a specific molecular event, and for this, expression of other immediate early response genes, such as c-fos and egr-1, was examined. Both c-Fos and Egr-1 were equally induced in HCT116 and HKe3 when the cells were stimulated with TPA (Fig. 1C), and induction reverted to basal levels 6 h after stimulation (data not shown). The transcriptional factor CREB binds the cAMP response element and activates transcription when it is phosphorylated at Ser-133 and in response to a variety of extracellular signals, including cAMP, increased intracellular Ca2+, and growth factors (30, 31). Phosphorylation of CREB on Ser-133 is equally induced in HCT116 and HKe3 when the cells were stimulated with TPA, and phosphorylated ATF1 was also induced (Fig. 1C). These observations suggest that specific molecular mechanisms are involved in suppression of the TPA-induced c-jun expression and phosphorylation at Ser-63 in HCT116 with activated Ki-ras.

**Suppression of TPA-induced Activation of SEK1-JNK Pathway in Human Colon Cancer Cells with Activated Ki-ras.** To determine whether the TPA-induced c-Jun phosphorylation at Ser-63 depends on JNK, the activity of JNK was examined. Both p46-JNK1 and p54-JNK2 were almost equally evident in HCT116 and HKe3 under these conditions (Fig. 2A). However, when these cells were stimulated by TPA, HKe3 showed strong phosphorylation on JNK1 and JNK2, whereas HCT116 showed little phosphorylation on these molecules (Fig. 2B). HKe3 cells also expressed a significantly higher phosphorylated JNK than did HCT116 cells when they were stimu-
Serum-starved; stimulation. Extracts were prepared at 1 h after stimulation.

\[ \text{MEK1/2} \text{Ser-217/Ser-221 antibody (B) parallel with the expression of phosphorylated c-Jun (Fig. 1, A).} \]

\[ \text{Antiphosphospecific JNK on Thr-183/Tyr-185 antibody (C), and antiphosphospecific MEK1/2 on Ser-217/Ser-221 antibody (D). Cells were serum starved for 24 h, followed by FCS or TPA stimulation. Extracts were prepared at 1 h after stimulation. Expo., exponentially growing; Starv., serum-starved; +TPA, TPA (50 ng/ml) stimulation; and P, phosphorylated form.} \]

Suppression of TPA-induced SEK1-JNK activation in HCT116. Western blot analysis with anti-JNK antibody (A), antiphosphospecific JNK on Thr-183/Tyr-185 antibody (B), antiphosphospecific SEK1 on Thr-223 antibody (C), and antiphosphospecific MEK1/2 on Ser-217/Ser-221 antibody (D). Cells were serum starved for 24 h, followed by FCS or TPA stimulation. Extracts were prepared at 1 h after stimulation. Expo., exponentially growing; Starv., serum-starved; +TPA, TPA (50 ng/ml) stimulation; and P, phosphorylated form.

Activated Ki-Ras Suppresses TPA-induced Activation of SEK1-JNK Pathway in Other Human Colon Cancer Cells. To exclude the possibility that suppression of TPA-induced activation of SEK1-JNK was due to clonality of HCT116 cells, other activated Ki-ras-disrupted clones, HK2–8 and HKh2, were examined (21). TPA-induced phosphorylation and activation of SEK1 and JNK were observed in both HK2-8 and HKh2, whereas HK2-10 (HCT116-derived clone) or HK2-6 (HCT116-derived wild-type Ki-ras-disrupted clone), did not show the phosphorylation of SEK1 or JNK (Fig. 5). Therefore, the activated Ki-Ras apparently suppresses the TPA-induced SEK1-JNK activation in HCT116 cells.

To confirm the relationship between activated Ki-ras and suppression of SEK1-JNK activation induced by TPA in human colon cancer cells, we examined another human colon cancer cell line, DLD-1, with a Gly-13→Asp mutation at Ki-ras. DK0-4 is a DLD-1-derived clone, in which activated Ki-ras was disrupted by gene targeting (21). DK0-4 cells showed SEK1 and JNK phosphorylation at Ser-219/Thr-223 and Thr-183/Tyr-185, respectively, when they were stimulated by TPA, whereas DLD-1 showed little phosphorylation of SEK1 or JNK on these activating sites (Fig. 5). These observations strongly suggest that activated Ki-Ras suppresses TPA-induced SEK1-JNK activation in human colon cancer cells.

Fig. 3. Time course of TPA-induced SEK1-JNK activation detected by Western blot with antiphosphospecific SEK1 and JNK antibodies. Cells were serum starved for 24 h, followed by TPA stimulation (50 ng/ml). Proteins were extracted at the indicated time points after stimulation.
Distinct PKC Isotypes Involved in TPA-induced JNK and ERK Activation in HCT116. To observe how PKC is involved in the SEK1-JNK activation in HKe3, when stimulated by TPA, PKC inhibitors H7 (26) and GF109203X (36) were used. HKe3 cells were serum starved for 24 h, followed by preincubation with H7 or GF109203X for 1 h before TPA stimulation, and the phosphorylation of SEK1, JNK, MEK1/2, and ERK1/2 was determined. The TPA-induced phosphorylation of MEK1/2 and ERK1/2 was reduced by H7, whereas this inhibitor did not reduce the phosphorylation of SEK1 or JNK, compared with that induced by TPA only (Fig. 6A; data not shown). On the other hand, GF109203X, a potent and selective inhibitor of PKC, completely inhibited the phosphorylation of SEK1 and JNK, at the concentration of 1 μM, whereas GF109203X (1 μM) only partially inhibited the phosphorylation of MEK1/2 and ERK1/2 (Fig. 6B). These results, taken together, suggest that the effect of PKC inhibitors will vary depending on the isotype of PKC and TPA-induced SEK1-JNK activation depends on the PKC isotype, distinct from that involved in MEK1/2-ERK activation in human colon cancer cells.

TPA-induced SEK1-JNK Activation Is Independent of PI3K Activation in Human Colon Cancer Cells. PI3K is a lipid kinase that phosphorylates phosphoinositides at the 3’ position of the inositol ring and has been implicated as a Ras effector (37, 38), and the ability of activated Ras to stimulate PI3K in addition to Raf is important in Ras transformation (39). To determine whether PI3K activation is involved in the TPA-induced SEK1-JNK activation in human colon cancer cells, an inhibitor for PI3K, LY294002, was used. HKe3 cells were serum starved for 24 h, followed by preincubation with various concentrations of LY294002 for 1 h before TPA stimulation. The phosphorylation of JNK and ERK1/2 were not inhibited by LY294002 at concentrations of 10, 50, or 100 μM (Fig. 7). The phosphorylation of SEK1 and MEK1/2 was not inhibited by LY294002 (data not shown). Thus, the TPA-induced SEK1-JNK and MEK1/2-ERK1/2 activation does not seem to occur through PI3K activation.

DISCUSSION

The evidence we obtained here clearly demonstrates that activated Ki-Ras-mediated signals are involved in the PKC-dependent SEK1-JNK pathway but not in the MEK1/2-ERK pathway in human colon cancer cells. MAPK are classified in three subfamilies: the ERK, JNK/SAPK, and p38 subfamilies (6, 7). Activation of ERK requires phosphorylation on both tyrosine and threonine residues, as catalyzed by dual-specificity enzymes known as MEK1/2. In parallel, another kinase cascade leading to activation of JNK/SAPK has been discovered, in which SEK1 exhibits high substrate affinity toward JNK/SAPK (27, 28, 40). Two distinct Ha-Ras-dependent MAPK exist: one is initiated by Raf-1, leading to ERK activation; the other is initiated by MEKK, leading to JNK activation (20). TPA is an efficient activator of ERK (9, 10, 33), functioning through activation of Raf-1 by PKC (41). TPA has little effect on JNK activity in fibroblasts or epithelial cells (33); however, TPA induced phosphorylation of JNK on both Thr-183 and Tyr-185 in the HCT116- and DLD-1-derived activated Ki-ras-disrupted clones, whereas the phosphorylation were not induced in HCT116 or DLD-1 (Figs. 2 and 5). Furthermore, the JNK phosphorylation coincided with c-Jun induction, c-Jun phosphorylation at Ser-63, and SEK1 phosphorylation at Ser-219/Thr-223 (Figs. 1 and 2), suggesting that TPA induced activation of the SEK1-JNK pathway, which, in the activated Ki-ras-disrupted clones, led to the activation of c-Jun.

Activation of MEK1 and MEK2 occurs through phosphorylation of two serine residues at positions 217 and 221. MEK activates ERK1 and ERK2 by phosphorylating both threonine and tyrosine residues. Although phosphorylation for MEK1/2 or for ERK1/2 phosphorylation for kinase activation was not observed under exponential growth or serum starvation conditions, TPA did induce both phosphorylation of MEK1/2 on Ser-217/Ser-221 and ERK1/2 at critical threonine and tyrosine residues for activation in both HKe3 and HCT116 (Fig. 2). This means that activated Ki-Ras is probably not involved in the MEK-ERK pathway.

p38 MAPK is activated by a variety of cellular stresses, including osmotic shock, inflammatory cytokines, lipopolysaccharides, UV, and growth factors (42–44). p38 MAPK is phosphorylated and activated at Thr-180/Tyr-182. p38 phosphorylation of Thr-180/Tyr-182 was equally induced by both H2O2 and UV in HCT116 and HKe3 (data not shown), which means that activated Ki-Ras is probably not involved in the regulating p38 activity.

JNK is activated by cellular stresses, such as UV irradiation (14, 18, 34, 45). When we asked whether activated Ki-Ras is involved in the UV-induced activation of JNK, phosphorylation of JNK at Thr-183/Tyr-185 was determined. UV-induced phosphorylation of JNK on Thr-183 and Tyr-185 was equally observed between HCT116 and HKe3 (data not shown). Anisomycin, which is known to activate JNK, also induced phosphorylation of JNK in both HCT116 and HKe3 (data not shown). Therefore, activated Ki-Ras does not seem to
suppress the UV-induced JNK activation and signaling pathways of activation of JNK differ between TPA and UV stimulation.

c-Jun is a major component of the AP-1 family of dimeric transcriptional factors, forming homodimers or heterodimers with other AP-1 families, including Jun, Fos, and ATF/CREB subfamilies. c-Jun shows numerous cellular phenotypes, such as oncogenic transformation, differentiation, the response to genotoxic agents, and apoptosis, through a combinatorial association with AP-1 families with a distinct DNA binding specificity (46, 47). The major regulators of the c-jun promoter are c-Jun and ATF2, acting as prebound dimers that are posttranslationally modified by JNK (48). Phosphorylation of ATF2 on Thr-71, which is a critical phosphorylation for ATF2 activation, was observed in HKe3 cells when they were stimulated with FCS or TPA but not in HCT116 cells, suggesting that TPA-induced JNK activation leads to activation of ATF2, which will induce c-jun induction in HKe3.

Oncogenic Ha-ras activates ERK2 without requiring growth factors, and this activation is constitutive and has two components, one of which depends on PKC activity whereas the other is independent (8). In Ha-ras-transformed cells, c-Jun overexpression was constitutive, whereas c-fos and c-myc expression was transient, and down-regulation of PKC led to a nonresponse to TPA-induced expression of c-fos. Among immediate response genes, such as c-jun, c-fos, egr-1, and c-myc, it is the c-jun gene that was not induced by TPA in human colon cancer cells with activated Ki-ras (Fig. 1; data not shown). The Raf-1-MEK-ERK pathway was also activated by TPA in HCT116 and HKe3 (Fig. 2; data not shown). A potent PKC inhibitor, H7, inhibited MEK1/2 activation in HKe3, whereas this inhibitor did not halt SEK1-JNK activation (Fig. 6A). On the other hand, GF109203X (1 μM) inhibited SEK1-JNK and MEK1/2-ERK1/2 activation, completely and partially, respectively (Fig. 6B). These results, taken together, suggest that activated Ki-Ras (Gly-13→Asp) is involved in the PKC-dependent SEK1-JNK pathway and that distinct PKC isotypes are involved in ERK and JNK activation in human colon cancer cells.

MEKK1, a serine-threonine kinase and an upstream activator of SEK, is a candidate effector of Ras (49, 50). MEKK1 has also been shown to bind to GTP-complexed Cdc42 and Rac1 (51), and mutationally activated Rac1 and Cdc42 GTPases potentially activate JNK, without affecting ERK (52). Furthermore, activation of PI3K by Cdc42 and Rac1 alters actin organization (53). However, the inhibitor for PI3K, LY294002 (53), did not inhibit TPA-induced SEK1-JNK activation in HKe3 (Fig. 7), suggesting that activation of PI3K, Rac1 or Cdc42 may not be involved in activated Ki-Ras-mediated SEK1-JNK signaling pathway in human colon cancer cells.

Ki-ras but not H- or N-ras was found to be essential for normal development in mice (54, 55), and Ki-Ras4B possesses distinct COOH-terminal modification (56), therefore, Ki-Ras will have specific functions in signal transduction that are not shared by other family member (49). Oncogenic Ha-Ras activates ERK whereas activated Ki-Ras suppresses JNK activation. We propose that the Ras family has a role in tumorigenesis by influencing activity governing the balance between ERK and JNK.
JNK. The cell lines used here are expected to be useful for elucidating functions of activated Ki-Ras in human colorectal tumorigenesis.

ACKNOWLEDGMENTS

We thank Mr. Ohara for critical comments on the manuscript.

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