Guanine-Nucleotide Exchange Protein C3G Activates JNK1 by a Ras-independent Mechanism

JNK1 ACTIVATION INHIBITED BY KINASE NEGATIVE FORMS OF MLK3 AND DLK MIXED LINEAGE KINASES

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Recently we have reported that the adaptor protein Crk transmits signals to c-Jun kinase (JNK) through C3G, a guanine-nucleotide exchange protein for the Ras family of small G proteins. Transient expression of C3G in 293T cells induced JNK1 activation without a significant effect on extracellular signal-related kinase 1 (ERK1), whereas mSos1 activated equally both JNK1 and ERK1. Coexpression of the dominant negative form of Ras-N17 did not suppress C3G-induced JNK1 activation but reduced the activity of JNK1 induced by mSos1, suggesting that Ras is not required for JNK activation by C3G. Ras-independent activation of JNK was supported by the finding that C3G-induced JNK activation was not inhibited by the dominant negative forms of Rac or Pak, which are components of the signaling pathway from Ras leading to JNK activation. In contrast, C3G-induced JNK1 activation was strongly inhibited by coexpression of the kinase negative forms of the mixed lineage kinase (MLK) family of proteins, MLK3 and dual leucine zipper kinase (DLK). In addition, MLK3-induced JNK1 activation was found to be suppressed by the kinase negative form of DLK, which bound to MLK3. These results suggest that C3G activates JNK1 through a pathway involving the MLK family of proteins.

C3G was originally isolated as a binding partner for the SH3 domain of Crk (1). The sequence homology of the catalytic domain of C3G suggested that C3G is a guanine-nucleotide exchange protein for the Ras family of small G proteins (1). In fact, C3G complements the lack of function of CDC25, a Ras guanine-nucleotide exchange protein in yeast (1), and in vitro studies demonstrated that it functions as an exchange factor for Ras family of proteins, Rap1 and R-Ras (2, 3). Although C3G binds Crk in various tissues (4, 5), the physiological relevance of the Crk-C3G complex is not clearly understood. We recently analyzed the activity of the mitogen-activated protein kinase (MAPK) family of proteins that includes extracellular signal-related kinase 1 (ERK1) and c-Jun N-terminal kinase 1 (JNK1) (6, 7) and found that C3G transmits signals to JNK1 in fibroblasts (8). However, the responsible small GTPase pathway that functions as a substrate for C3G and transmits signals to JNK remains unidentified.

The small GTPase, Ras, has been reported to transmit a range of signals in response to various stimuli, including a well-established pathway to ERK activation for cell cycle progression (9, 10). Sequential activation of serine/threonine kinases such as c-Raf-1 and MAPK/ERK kinase 1 (MEK1) downstream of Ras has been shown to be involved in ERK activation (11). In addition, recent reports have shown that Ras also activates JNK via the Rho family of small G proteins, Rac or Cdc42 (12, 13) through sequential activation of serine/threonine kinases, p21-activated kinase 1 (Pak1), MEK1 kinase 1 (MEK1), and a direct activator of JNK, Sek1/MK4 (14–17). Because C3G has been shown both to activate Ras in yeast (2) and to weakly increase the active form of H-Ras when this was incubated with C3G in vitro (3), in the present study we have examined the possibility that Ras may play a critical role in C3G-mediated signal transduction in JNK activation.

MATERIALS AND METHODS

Plasmids—Mammalian expression plasmids, pCAGGS-C3G, pCAGGS-C3G-F, pCAGGS-mSos1, and pCAGGS-mSos1-F were gifts from Michiyuki Matsuda (NIH, Japan), pcDNA3-HA-JNK1, pcEF-GST-Pak-N, pcDNA3-Ras-N17, pCEV29-RhoA-N19, pCEF-MLK3, and pCEF-MLK3-K114R were from Silvio J. Gutkind (NIH). pCEP-EE-Rap-N17 was from Laurence Quilliam, pEF-BOS-HA-Rac-N17 and pEF-BOS-HA-Cdc42-N17 were from Koizo Kiyohara (NAIST, Japan), pcDNA3-Flag-DLK and pcDNA3-Flag-DLK-K185A were from Lawrence F. Holzmann (University of Michigan), and pEBG-Sek-1 and pEBG-Sek-1-KR were from Leonard I. Zon (Harvard University). Expression plasmids for ΔMEKK1 and ΔMEKK1-KR were constructed using pFC-MEKK (Stratagene). The expression plasmid for TC21/Ras2-N17 was from Andrew M. Chang (Mount Sinai Medical School, New York).

Antibodies—Anti-C3G (C-19), anti-Sos1/2 (D-21), anti-Rac1 (C-14), anti-Cdc42Hs (P1), anti-JNK1 (C-17), and anti-MLK3 (C18) antibodies were purchased from Santa Cruz Inc. Anti-p21-Ras antibody (Ab-3), anti-HA antibody, and anti-Flag antibody (M2) were obtained from Calbiochem, Boehringer Mannheim, and Kodak, respectively.

Transient Transfection of Plasmid DNA—293T cells were cultured in Dulbecco’s modified Eagle’s medium with 10% calf serum using a plastic dish 60 mm in diameter, and DNAs were introduced into cells using a modified calcium-phosphate transfection system (Stratagene). The procedures of cell lysis and immunoblotting were described elsewhere (12).

In Vitro JNK and ERK Kinase Assays—JNK and ERK kinase activities were measured by in vitro kinase assay described elsewhere using GST-c-Jun (amino acids 1–79) and myelin basic protein, respectively, as substrates (12). Incorporated radio isotope activities with each substrate were measured, and the results are shown as bar graphs.

RESULTS AND DISCUSSION

C3G and Sos Have Different Specificities toward ERK1 and JNK in 293T Cells—Because Ras in its GTP bound state can activate JNK via a Rac, Cdc42, Pak, MEKK1, Sek1/1MKK4 signaling cascade, we wanted to investigate whether C3G activated JNK via this pathway. To evaluate the effects of C3G on Ras, we measured the potential of C3G to activate the MAPKs, JNK, c-Jun N-terminal kinase, MLK, mixed lineage kinase, ERK, extracellular signal-related kinase, MEK, MAPK/ERK kinase, MEKK, MEK kinase, Pak, p21-activated kinase, DLK, dual leucine zipper kinase, GST, glutathione S-transferase, M KK, MAPK kinase.
in ERK1 or JNK1 activity compared with the cytoplasmic derivatives (Fig. 1A). It is conceivable that high levels of C3G or mSos1 expression in the transient expression system may permit easy contact with their substrates localized in the vicinity of the membrane, and thus farnesylation does not result in further increases in kinase activation. Therefore we used wild type C3G and mSos1 for further analysis of the downstream signaling pathway of C3G-induced JNK activation.

Expression of the Dominant Negative Form of Ras Suppressed mSos1-induced, but Not C3G-induced, Activation of JNK1 in 293T Cells—A reciprocal experiment to test whether C3G-dependent activation of JNK is regulated by Ras involves dominant negative Ras-N17 protein. Although coexpression of Ras-N17 significantly reduced mSos1-induced JNK activation, cotransfection of Ras-N17 did not have any effect on C3G-induced JNK1 activation even on the mild 2.5-fold activation resulting from transfection of reduced amounts of C3G (Fig. 2A). In agreement with Fig. 1, these data suggest that C3G activates JNK1 in a Ras-independent manner. C3G has been reported to activate Rap1 in an in vitro guanine-nucleotide exchange assay (2). Therefore, we tested whether a dominant negative form of Rap1, Rap-N17, can suppress C3G-induced JNK activation. As shown in Fig. 2B, the coexpression of Rap1-N17 did not suppress either C3G or mSos1-induced JNK activation, suggesting that Rap1 is also not involved in JNK activation. In addition, N17 TC21/R-Ras2, a dominant negative form of another member of the Ras family of proteins, did not inhibit C3G-induced JNK1 activation (Fig. 2C).

FIG. 1. In vitro kinase assays for JNK1 and ERK1 in 293T cells expressing C3G or mSos1. A, 4 μg of mammalian expression plasmids pCAGGS for C3G, C3G-F, mSos1, or mSos1-F were cotransfected with either 2 μg of expression plasmid for HA-JNK1 or GST-ERK1 into 293T cells for the JNK1 or ERK1 assays, respectively, UV irradiation and c-Ha-Ras-V12 were employed as positive controls in the JNK1 and ERK1 assays, respectively. The averages of radioisotope counts from three independent experiments are shown as a bar graph. The open bars show the results of the JNK1 assay, and the closed bars show those of the ERK1 assays. B, a typical result of the JNK1 assay employing GST-c-Jun (1–79) is shown at the top. Protein expression levels of HA-JNK1, C3G, and mSos1 were confirmed by immunoblotting (IB) with anti-HA, C3G, and Sos antibodies using total cell lysates containing 20 μg of protein/lane. Protein expression in each lane is as follows: vector control (lane 1), HA-JNK1 (lane 2), C3G and HA-JNK1 (lane 3), C3G-F and HA-JNK1 (lane 4), mSos1 and HA-JNK1 (lane 5), mSos1-F and HA-JNK1 (lane 6), and HA-JNK1 and UV irradiation (lane 7). C, a typical result of the ERK1 assay using myelin basic protein (MBP) is displayed at the top. Protein expression levels of GST-ERK1, C3G, mSos1, and H-Ras were confirmed by immunoblotting (IB) with anti-HA, C3G, Sos, and pan-Ras antibodies. Protein expression in each lane is as follows: control (lane 1), GST-ERK1 (lane 2), C3G and GST-ERK1 (lane 3), C3G-F and GST-ERK1 (lane 4), mSos1 and GST-ERK1 (lane 5), mSos1-F and GST-ERK1 (lane 6), and GST-ERK1 and H-Ras (lane 7).

JNK1, and ERK1 and compared this with mSos1, which is an authentic activator for Ras. cDNAs for C3G or mSos1 were transiently coexpressed with either epitope tagged JNK1 or ERK1 in human embryo kidney 293T cells. JNK1 or ERK1 activity was then measured by an in vitro kinase assay using either GST-c-Jun or myelin basic protein as substrates. As shown in Fig. 1A, C3G preferentially activated JNK1 compared with ERK1 when compared with the positive controls, which were UV irradiation or Ras-V12 expression. In contrast, mSos1 activated both JNK1 and ERK1 to a similar extent. The protein expression levels of each molecule in the JNK and ERK kinase assays were examined by immunoblotting and are shown in Fig. 1 (B and C, respectively). The results suggested that Ras may not be involved in the C3G-induced activation of JNK1.

We also tested the effects of membrane targeted forms of C3G or mSos1 using a farnesylation-acceptabile sequence on JNK1 or ERK1 activation in a transient protein expression system, because it is known that stably expressed Sos-F strongly activates ERK1 in murine fibroblasts compared with wild type mSos1 (Ref. 18 and data not shown). Unlike the results obtained in the stable protein expression system, we found that the presence of a farnesylation targeting sequence on either C3G or mSos1 did not result in a significant increase
2.6. Immunoblotting are shown (IB by immunoblotting are also shown (IB samples. Representative results of the JNK assay are shown at the graph. B, a kinase negative form of MLK3 suppressed C3G-induced JNK1 activation. 4 μg of plasmid for MLK3-K114R was cotransfected with plasmids for C3G, Cdc42-V12, or ΔMEKK1 as indicated by + or − below the graph. In A and B, 2 μg of HA-JNK1 was cotransfected in all samples. Representative results of the JNK assay are shown at the top graph (GST-J c-Jun (1–79)). Protein expression levels of HA-JNK1 as detected by immunoblotting are also shown (IB). C, verification of JNK1 activation by MLK3 and DLK. 4 μg of expression plasmid for MLK3 or DLK is cotransfected with HA-JNK1, and measured JNK activity is shown in the top panel. Protein expression in each lane is as follows: vector control (lanes 1 and 2), MLK3 (lane 3), MLK3-K114R (lane 4), Flag-DLK (lane 5), and Flag-DLK-K185A (lane 6). HA-JNK1 is expressed in lanes 2–6. Expression levels of MLK3, DLK, and HA-JNK1 as detected by immunoblotting are shown (IB).

Suppression of C3G-induced JNK1 Activation by the kinase negative form of MLK3-K114R in 293T cells. A, a kinase deletion form of Pak1 (Pak-N) did not suppress C3G-induced JNK1 activation. 4 μg of plasmid for GST-Pak-N was cotransfected with 4 μg of plasmids for C3G or Cdc42-V12 as indicated by + or − below the graph. B, a kinase negative form of MLK3 suppressed C3G-induced JNK1 activation. 4 μg of plasmid for MLK3-K114R was cotransfected with plasmids for C3G, Cdc42-V12, or ΔMEKK1 as indicated by + or − below the graph. In A and B, 2 μg of HA-JNK1 was cotransfected in all samples. Representative results of the JNK assay are shown at the top graph (GST-J c-Jun (1–79)). Protein expression levels of HA-JNK1 as detected by immunoprecipitation using anti-Flag antibody followed by immunoblotting with anti-MLK3 antibody (top panel). Interaction of MLK3 and Flag-DLK-K185A in 293T cells. Expression proteins in each lane are as follows: vector control (lane 1), MLK3 (lane 2), Flag-DLK-K185A (lane 3), and Flag-DLK-K185A (lane 4). Protein expression levels of MLK3 were confirmed by anti-MLK3 antibody using total cell lysates containing 20 μg of protein (top panel). Interaction of MLK3 and Flag-DLK-K185A was examined by immunoprecipitation using anti-Flag antibody following immunoblotting with anti-MLK3 antibody (middle panel). The same filter was reprobed with anti-Flag antibody to confirm the amount of precipitated Flag-DLK-K185A (bottom panel).

negative form of MLK3-K114R suppressed C3G-induced JNK1 activation (Fig. 3B). In these experiments, we used Cdc42-V12 and a truncated form of ΔMEKK1 that has been shown to activate JNK1 as positive and negative controls, respectively, for the estimation of the dominant negative effect of MLK3 (Fig. 3B), because MLK3 has been shown to function between Cdc42 and MEKK1 (28). In control experiments (Fig. 3C) JNK activation could only be observed with native MLK3 but not kinase negative MLK3. The results (Fig. 3, A and B) suggest the possible involvement of MLK3 rather than Pak1 in C3G-induced JNK1 activation.

Suppression of C3G-induced JNK1 Activation by Kinase Negative Form of DLK—Because MLK3 has been shown to transmit signals to MEKK1 (28), we examined a role for...
MEKK1 in C3G-dependent JNK1 activation. As shown in Fig. 4A, expression of the kinase negative form of MEKK1-KR attenuated by approximately 50% the C3G-induced JNK1 activation. Because the suppression of C3G-induced JNK1 activation by MEKK1-KR was partial, it is possible that another molecule functions as MAPK kinase for JNK1 instead of MEKK1. Because one of the MLK family of proteins, DLK, has been shown to function as a MEKK1-like protein (29), we examined a possible role for DLK and found that coexpression of kinase negative form of DLK-K185A clearly suppressed the C3G-induced JNK1 activation in 293T cells (Fig. 5). We could also demonstrate that wild type DLK can activate JNK1 in 293T cells (Fig. 5).

Because C3G-induced JNK1 activation was suppressed by dominant negative forms of both MLK3 and DLK, we examined whether MLK3 activates JNK through DLK in 293T cells. Because MLK3 has been reported to function as a Pak1-like protein (28) and DLK as a MEKK1-like protein (29), it is likely that MLK3 functions upstream of DLK. We found that the kinase negative form of DLK-K185A strongly suppressed the MLK3-induced JNK1 activation (Fig. 4C). We could also demonstrate the interactions of overexpressed MLK3 and DLK-K185A in 293T cells (Fig. 4D). Although the mechanisms of the interactions of MLK3 and DLK are unknown, this association may contribute to the suppression of MLK3-induced JNK1 activation by DLK-K185A. The data suggest that both MLK3 and DLK may be involved in the signaling pathway in C3G-induced JNK1 activation in 293T cells (Fig. 5).

Our present studies suggest that C3G-mediated signaling pathway to JNK1 occurs in a Ras-independent mechanism. Consistent with this idea, Rac, which is a major signal transducer for Ras to JNK1, does not have any role in C3G-induced JNK1 activation. Furthermore, we have shown that another member of the Ras family of proteins Rap1 does not induce JNK1 activation downstream of C3G. Downstream signaling of Rap1 may involve the activation of ERK1 through B-Raf under cAMP stimulation (30). Recently, R-Ras has been shown to be activated by both C3G and Ras-GTP/Cdc25Mo in vitro assays (3), and it has been reported that R-Ras could transmit signals to Akt through phosphatidylinositol 3-kinase but not to ERK and JNK (31). We are currently investigating the small GTPase that may serve as a substrate of C3G and at the same time induce JNK1 activation. In this study, we have examined the involvement of serine/threonine kinases in C3G-induced JNK activation and clearly showed that kinase negative forms of the MLK family of proteins, MLK3 and DLK, suppress C3G-induced JNK1 activation in 293T cells. Although one should be cautious about the possibility that a dominant negative form of kinases transiently overexpressed in cells could block the function of related family of proteins, our observations strongly suggest a possible role for the MLK family of proteins in C3G signaling to JNK1.

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