

## Crk Activation of JNK via C3G and R-Ras\*

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Naoki Mochizuki‡, Yusuke Ohba‡, Shin Kobayashi‡, Naomi Otsuka‡, Ann M. Graybiel§, Shinya Tanaka¶, and Michiyuki Matsuda‡¶

From the ‡Department of Pathology, Research Institute, International Medical Center of Japan, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan, the §Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and the ¶Department of Pathology, Hokkaido University School of Medicine, Sapporo 060-8638, Japan

**v-crck** is an oncogene identified originally in CT10 chicken tumor virus. C3G, a guanine nucleotide exchange factor (GEF) for Rap1 and R-Ras, is postulated to transduce the oncogenic signal of v-Crk to c-Jun kinase (JNK). We have found that R-Ras, but not Rap1, mediates JNK activation by v-Crk in 293T and NIH 3T3 cells. Constitutively activated R-Ras, R-Ras<sup>Val-38</sup>, but not Rap1<sup>Val-12</sup>, activated JNK, as did the constitutively active H-Ras<sup>Val-12</sup> or Rac1<sup>Val-12</sup>. v-Crk activation of JNK was inhibited by a dominant-negative mutant of R-Ras, R-Ras<sup>Asn-43</sup>. JNK activation by R-Ras<sup>Val-38</sup> was inhibited by a dominant-negative mutant of mixed lineage kinase 3. Among six GEFs for Ras-family G proteins, mSos1, Ras-GRF, C3G, CalDAG-GEFI, Ras-GRP/CalDAG-GEFII, and Epac/cAMP-GEFI, GEFs for either H-Ras or R-Ras activated JNK and c-Jun-dependent transcription. CalDAG-GEFI and Epac/cAMP-GEFI, both of which are GEFs specific for Rap1, did not activate JNK or c-Jun-dependent transcription. These results demonstrate that R-Ras, but not Rap1, is the downstream effector of C3G to stimulate JNK. Finally, we found that expression of the dominant-negative R-Ras mutant induced flat reversion of NIH 3T3 cells transformed by v-Crk, suggesting that R-Ras-dependent JNK activation is critical for the transformation by v-Crk.

v-Crk was isolated originally as an oncogene product of CT10 chicken tumor virus and consisted mostly of the SH2<sup>1</sup> and SH3 domains (1). Cells infected by CT10 accumulate several phosphotyrosine-containing proteins that bind to the SH2 domain of v-Crk and serve as plasma membrane anchors for v-Crk (2). The SH3 domain of v-Crk and its cellular homologs, CrkI and CrkII, bind to several proteins that have proline-rich sequences (2). Among them, C3G, a guanine nucleotide exchange factor (GEF) for Rap1 and R-Ras (3) and DOCK180 are the major target proteins, as judged from the results of far-Western blotting with Crk SH3 used as a probe (2).

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¶ To whom correspondence should be addressed: Dept. of Pathology, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan. Fax: 81-3-3205-1236; E-mail: mmatsuda@ri.imcj.go.jp.

<sup>1</sup> The abbreviations used are: SH, Src homology; GEF, guanine nucleotide exchange factor; JNK, c-Jun kinase; GST, glutathione S-transferase; PI 3-kinase, phosphoinositide 3-kinase; PAGE, polyacrylamide gel electrophoresis; MLK, mixed lineage kinase.

Transformation of NIH 3T3 cells by v-Crk is enhanced by the overexpression of C3G and inhibited by a catalytically inactive C3G mutant (4). C3G activates c-Jun kinase (JNK), as does v-Crk. The dominant-negative mutant of Sek1, a direct upstream activator of JNK, also inhibits transformation of NIH 3T3 cells by v-Crk, indicating that JNK activation is, at least, required for the transformation by v-Crk (4).

C3G promotes the guanine nucleotide exchange reaction of Rap1 and R-Ras (5, 6). There are two Rap1 proteins, Rap1A and Rap1B, in mammalian cells; however, no functional difference has been known (7). Rap1, which shares 55% identity in the amino acid sequence with H-Ras, was isolated as a suppressor of Ras in NIH 3T3 cells and was subsequently shown to compete with Ras in Raf activation (7). Interestingly, Rap1 has been shown to transform Swiss 3T3 cells, suggesting that the effect of Rap1 on oncogenesis may change drastically in a cellular milieu (8).

cDNA of R-Ras, another member of Ras family GTPase, was isolated by low-stringency hybridization with a v-H-ras probe (9). Constitutively active R-Ras does not induce morphological transformation of NIH 3T3 cells (10–12) or rat fibroblasts (13). However, cells expressing the active R-Ras mutant grow in low serum and in athymic mice (11–14), indicating that R-ras is also an oncogene. R-Ras activates PI 3-kinase, but not Raf (10, 15). Thus, R-Ras seems to transform cells in a pathway distinct from the Raf-ERK/MAPK pathway. In this study, we have examined whether Rap1 or R-Ras plays the principal role in the activation of JNK by C3G.

### EXPERIMENTAL PROCEDURES

**Plasmids**—Wild-type and dominant-negative mutant of Rap1A, pSRα-Krev-1–17N, were obtained from M. Noda at Kyoto University (16). The wild-type, constitutively active, and dominant-negative mutants of R-Ras, pEXV-R-Ras, pEXV-R-RasV38, and pEXV-R-RasN43, respectively, were obtained from A. Hall (17). pCEV-c-Ha-ras<sup>Val-12</sup>, pCEV-c-Ha-ras<sup>Asn-17</sup>, pEF-Bos-Cdc42<sup>Val-12</sup>, and pEF-Bos-Rac<sup>Val-12</sup> were obtained from K. Kaibuchi (NAIST, Japan). Coding regions of these Ras family GTPases were amplified by polymerase chain reaction and subcloned into pCXN2-Flag and pCAGGS-Hyg (18). pEBG-JNK and pGEX-c-Jun were obtained from B. J. Mayer (19). pMEX-v-Crk encodes the viral Crk oncoprotein (4). pCAGGS-C3G, pCAGGS-C3G-F, pCAGGS-C3G-CD, pCAGGS-Sos, pCAGGS-RasGRF, pCMV-CalDAG-GEFI, pCAGGS-CalDAG-GEFII, and pCMV-cAMP-GEFI have been described previously (5, 20–22). pCEFL-MLK3-K114R was obtained from J. S. Gutkind (4).

**Antibody**—Rabbit antisera against C3G and GST were developed in our laboratory (3, 23). Anti-FLAG M5 monoclonal antibody and anti-Crk monoclonal antibody were purchased from Sigma and Transduction Laboratories, respectively. Anti-RasGRF, anti-mSos1, and anti-MLK3 antibodies were from Santa Cruz Biotech.

**Cell Culture and Transfection**—293T cells were cultured in Dulbecco's modified Eagle's medium (Nissui, Tokyo) supplemented with 10% fetal calf serum and transfected by the calcium phosphate method. NIH 3T3 and COS1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and transfected by Fu-

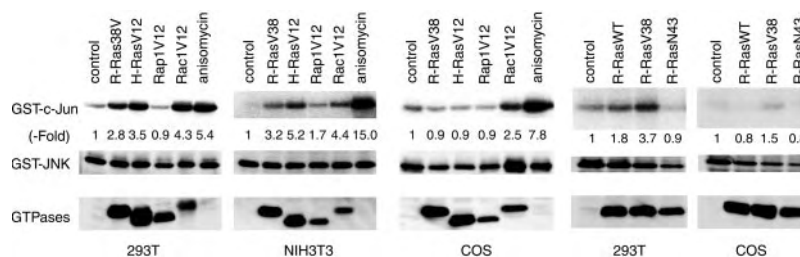


FIG. 1. **Activation of JNK by R-Ras.** 293T, NIH 3T3, or COS cells as indicated at the *bottom* were transfected with the expression vectors for the FLAG-tagged GTPases indicated on the *top* and for GST-JNK. Where indicated, cells were treated with anisomycin for 10 min before cell lysis. JNK activities were measured by *in vitro* kinase assay with GST-c-Jun (1–79) used as a substrate. -Fold activation compared with the control is indicated at the *bottom* of the first column. Total lysates were analyzed by immunoblotting with anti-GST for GST-JNK and anti-FLAG antibodies for GTPases.

Gene6 (Roche Molecular Biochemicals). Twenty-four hours after transfection, cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 5  $\mu$ g/ml aprotinin) and cleared by centrifugation at  $10,000 \times g$  for 15 min. Aliquots of the total cell lysates were analyzed by SDS-PAGE and immunoblotting. From the remaining lysates, GST-JNK was collected on glutathione-Sepharose and used for *in vitro* JNK assay. As a positive control, cells were stimulated with 100 ng/ml anisomycin (Sigma) for 10 min before cell lysis.

**In Vitro JNK Assay**—JNK kinase activity was measured by *in vitro* kinase assay with GST-c-Jun (amino acids 1–79) used as substrate (24). After SDS-PAGE, gels were analyzed by a BAS 1000 image analyzer (Fuji film, Tokyo).

**Activation of c-Jun Transcription Factor**—Activation of the c-Jun transcriptional factor was assayed by use of a PathDetect kit (Stratagene).  $2 \times 10^5$  of 293T cells were transfected with 1  $\mu$ g of pFR-Luc monitor plasmid, 50 ng of pFA2-c-Jun activator plasmid, and 1  $\mu$ g of expression vectors for GEFs of Ras family GTPases. Twenty-four hours after transfection, cells were lysed in lysis buffer, and luciferase activity was measured by a LAS 1000 image analyzer (Fuji film).

**Morphological Reversion of v-crk-transformed Cells by Expression of the Dominant-negative R-Ras**—v-Crk-NIH 3T3 cell (clone 1-1) has been described previously (4). The v-Crk-NIH 3T3 cells were transfected with pCAGGS-Hyg, pCAGGS-Hyg-Flag-R-Ras<sup>Asn-43</sup>, or pCAGGS-Hyg-Flag-H-Ras<sup>Asn-17</sup> with LipofectAMINE (Life Technologies, Inc.). After 2 days, cells were selected in the presence of hygromycin B (Roche Molecular Biochemicals). Ten days later, morphology of the colonies were observed and flat revertants were counted under the microscope as described (16). Several representative flat revertants and spindle non-revertants were isolated and analyzed by SDS-PAGE and immunoblotting.

## RESULTS

**Activation of JNK by R-Ras**—C3G promotes a guanine nucleotide exchange reaction of Rap1 and R-Ras (5, 6). We first tested whether constitutively active mutants of Rap1 and R-Ras, Rap1<sup>Val-12</sup>, and R-Ras<sup>Val-38</sup>, respectively, stimulated JNK in 293T, NIH 3T3, and COS cells. As shown in Fig. 1, R-Ras<sup>Val-38</sup>, but not Rap1<sup>Val-12</sup>, activated JNK, as did active H-Ras and Rac in 293T and NIH 3T3 cells. Previously, it was reported that R-Ras did not activate JNK in COS cells (15). In concordance with this observation, we found that H-Ras<sup>Val-12</sup> or R-Ras<sup>Val-38</sup> did not significantly activate JNK in COS cells, whereas Rac<sup>Val-12</sup> did so efficiently. The activation of JNK in 293T cells was observed most prominently by use of the GTPase-deficient active form, R-Ras<sup>Val-38</sup>, and slightly by the wild-type R-Ras. The dominant-negative mutant, R-Ras<sup>Asn-43</sup>, did not stimulate JNK.

**Inhibition of JNK Activation by Dominant-negative R-Ras**—We next examined whether the dominant-negative mutants of H-Ras, R-Ras, and Rap1 inhibited C3G-dependent activation of JNK. As shown in Fig. 2A, R-Ras<sup>Asn-43</sup> partially inhibited the C3G-dependent JNK activation. Neither H-Ras<sup>Asn-17</sup> nor Rap1<sup>Asn-17</sup> inhibited JNK activation by C3G. The inability of Rap1<sup>Asn-17</sup> to inhibit JNK activation may partly be due to the low expression level of Rap1<sup>Asn-17</sup> and partly due to its inability to interact with C3G (25). The incomplete inhibition by R-Ras<sup>Asn-43</sup> may suggest that C3G has multiple path-

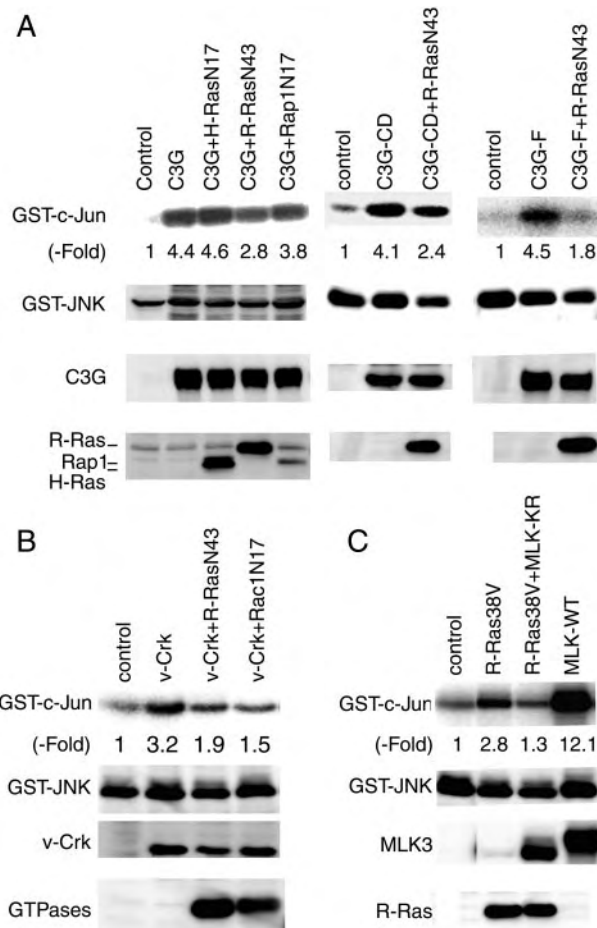
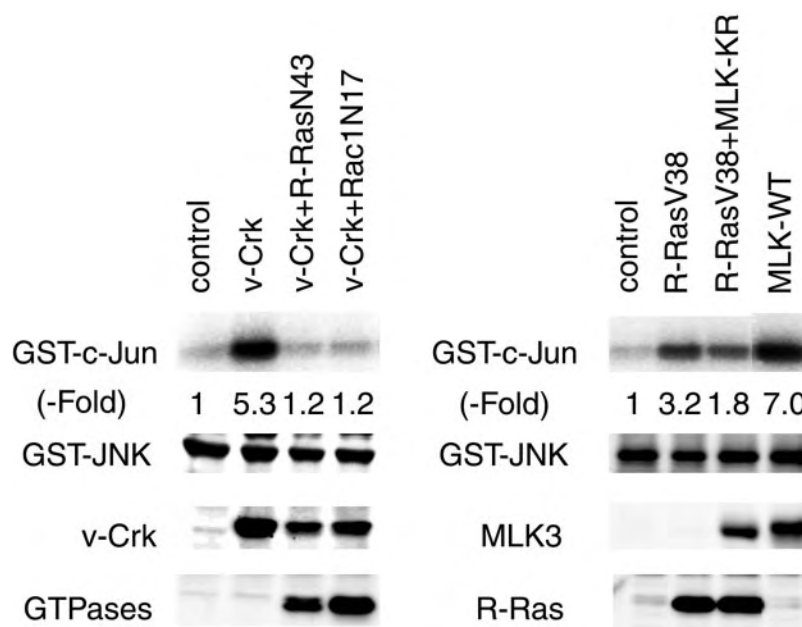


FIG. 2. **Inhibition of JNK activation by a dominant-negative R-Ras mutant.** A–C, 293T cells were transfected with the expression vectors for the proteins indicated at the *top*. JNK activities were measured by *in vitro* kinase assay with GST-c-Jun(1–79) used as a substrate. -Fold activation compared with the control is indicated at the *bottom* of the first column. Total lysates were analyzed by immunoblotting with anti-GST antibody, anti-C3G antibody, anti-FLAG antibody for the GTPases, or anti-MLK3 antibody.

ways leading to the activation of JNK. Thus, we examined the effect of R-Ras<sup>Asn-43</sup> on the JNK activation by two C3G active mutants, C3G-CD and C3G-F (Fig. 2B). C3G-CD lacks the amino-terminal negative regulatory region. C3G-F is fused to the CAAX box of K-Ras and activated by membrane translocation. In either case, R-Ras<sup>Asn-43</sup> inhibited JNK activation, but again, partially. Because C3G-CD consists solely of the catalytic domain, it is unlikely that C3G-CD activated JNK without activation of Rap1 or R-Ras. In addition, C3G- $\Delta$ CD, which lacks

FIG. 3. **JNK activation in NIH 3T3 cells.** NIH 3T3 cells were transfected with the expression vectors for the proteins indicated at the top. JNK activities were measured by *in vitro* kinase assay with GST-c-Jun(1–79) used as a substrate. -Fold activation compared with the control is indicated at the bottom of the first column. Total lysates were analyzed by immunoblotting with anti-GST antibody, anti-Crk antibody, anti-FLAG antibody for the GTPases, or anti-MLK3 antibody.



the catalytic domain, did not activate JNK.<sup>2</sup> Thus, the incomplete inhibition by R-Ras<sup>Asn-43</sup> may be ascribable to the overexpression of C3G or to the low affinity of R-Ras<sup>Asn-43</sup> to C3G.

We next tested the effect of the dominant-negative mutants of R-Ras and Rac on the v-Crk dependent JNK activation in 293T cells. As shown in Fig. 2B, v-Crk dependent JNK activation was inhibited by the expression of R-Ras<sup>Asn-43</sup> mutant. Rac1<sup>Asn-17</sup> mutant also inhibited v-Crk-dependent JNK activation as reported previously (26).

C3G-dependent activation of JNK is independent of Ras and dependent on MLK3 (27). To confirm that C3G activates JNK via R-Ras, we examined whether R-Ras also requires MLK3 for JNK activation by the use of the dominant-negative MLK3 mutant. As shown in Fig. 2C, the R-Ras dependent JNK activation was inhibited by the dominant-negative MLK3 (Fig. 2C), suggesting that C3G-dependent JNK activation is mediated by R-Ras and MLK3.

**Inhibition of JNK Activation by the Dominant-negative R-Ras in NIH 3T3 Cells**—v-Crk requires C3G-dependent JNK activation for the transformation of NIH 3T3 cells (4, 27). Thus, we examined whether R-Ras is involved in the JNK activation by v-Crk in NIH 3T3 cells. As shown in Fig. 3, JNK activation by v-Crk was inhibited by R-Ras<sup>Asn-43</sup> and Rac1<sup>Asn-17</sup>, suggesting that in NIH 3T3 cells both R-Ras and Rac were required for the optimal JNK activation by v-Crk. R-Ras-induced JNK activation was inhibited by the dominant-negative MLK, MLK-KR, in NIH 3T3 cells as we observed in 293T cells. Thus, we concluded that R-Ras was required for the JNK activation by v-Crk in NIH 3T3 cells as in 293T cells.

**Activation of JNK and c-Jun-dependent Transcription by the Guanine Nucleotide Exchange Proteins**—To exclude the involvement of Rap1 from the C3G-dependent activation of JNK, we utilized two guanine nucleotide exchange proteins for Rap1, CalDAG-GEFI and Epac/cAMP-GEFI (21, 22). Both promote a guanine nucleotide exchange reaction of Rap1, but not R-Ras. For comparison, we used three other GEFs for Ras family proteins, RasGRF and RasGRP/CalDAG-GEFII, which activate both Ras and R-Ras, and Sos, which activates only Ras. Neither CalDAG-GEFI nor Epac/cAMP-GEFI activated JNK (Fig. 4, A and B). We further confirmed that GEFs for Rap1 did not activate JNK by measuring the transcriptional activity of c-

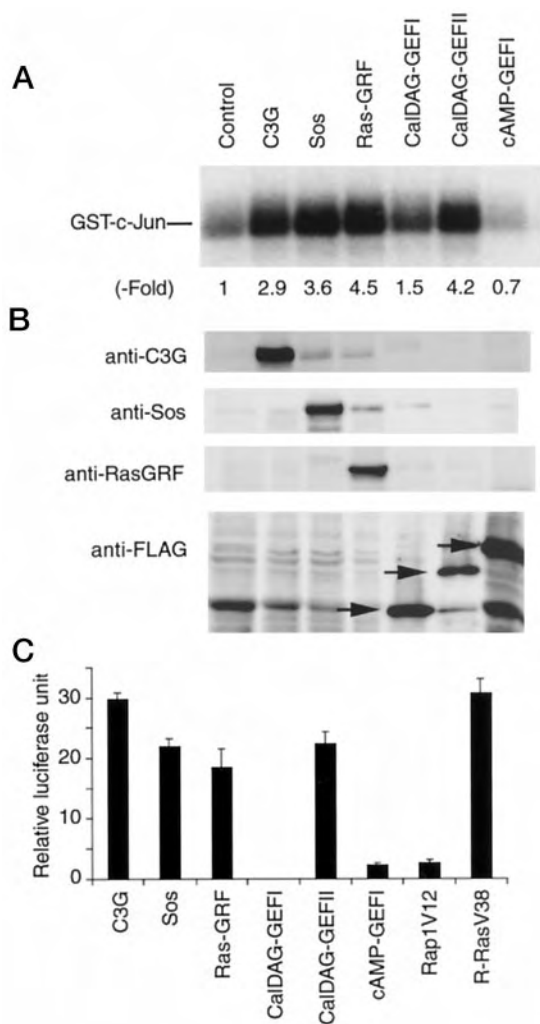
Jun-GAL4 chimeric protein (Fig. 4C). Rap1<sup>Val-12</sup> and GEFs specific for Rap1, CalDAG-GEFI, and Epac/cAMP-GEFI, did not activate c-Jun-dependent transcription. Other GEFs, C3G, Sos, RasGRF, and Ras-GRP/CalDAG-GEFII, activated c-Jun-dependent transcription, as did R-Ras<sup>Val-38</sup>. This result strongly suggests that R-Ras, but not Rap1, mediated JNK activation by C3G. Both Ras-GRF and Ras-GRP/CalDAG-GEFII activate Ras and R-Ras; therefore, these GEFs appear to have two pathways for activation of JNK. In addition, it is noteworthy that Sos activates Rac by its Dbl-homology domain (28); therefore, Rac-dependent JNK activation may also account for the Sos activation of JNK.

**Morphological Reversion of v-crk-transformed Cells by Expression of the Dominant-negative R-Ras**—Finally, we examined whether the dominant-negative R-Ras mutant, R-Ras<sup>Asn-43</sup>, reverted v-crk-induced transformation of NIH 3T3 cells. Sixteen percent of the stable transfectants of R-Ras<sup>Asn-43</sup> were flat and scored as revertants (Fig. 5A). Most of these revertants displayed a peculiar flat polygonal shape with large cytoplasm as shown in Fig. 5B. From the R-Ras<sup>Asn-43</sup>-transfected colonies, several flat revertants and spindle non-revertants were isolated and subjected to immunoblotting (Fig. 5C). Cells from flat colonies expressed R-Ras<sup>Asn-43</sup>, whereas spindle cells expressed little or no R-Ras<sup>Asn-43</sup> mutants. Flat revertants appeared also by transfection of control vector (Fig. 5A); however, the morphology of these cells were clearly distinguishable from those expressing R-Ras<sup>Asn-43</sup>.<sup>2</sup>

#### DISCUSSION

Previously, Dolfi *et al.* (29) demonstrated that integrin stimulation activates JNK in a manner dependent on Crk and C3G. C3G is rapidly phosphorylated on tyrosine upon integrin-mediated cell adhesion (30), and tyrosine phosphorylation of C3G stimulates its catalytic activity (31). In this report, we have shown that R-Ras, but not Rap1, is the downstream effector of C3G for the activation of JNK. In concordance with this finding, there is a resemblance in the effect of Crk and R-Ras on cell migration. Cell migration is enhanced by the expression of Crk (32, 33) or R-Ras (34, 35). The constitutively active R-Ras increases the ligand-binding affinity of integrin (36, 37); similarly, expression of CrkL activates cell adhesion of 32D cells (38). This adhesion of 32D cells was enhanced by the expression of C3G and inhibited by the dominant-negative R-Ras. Alto-

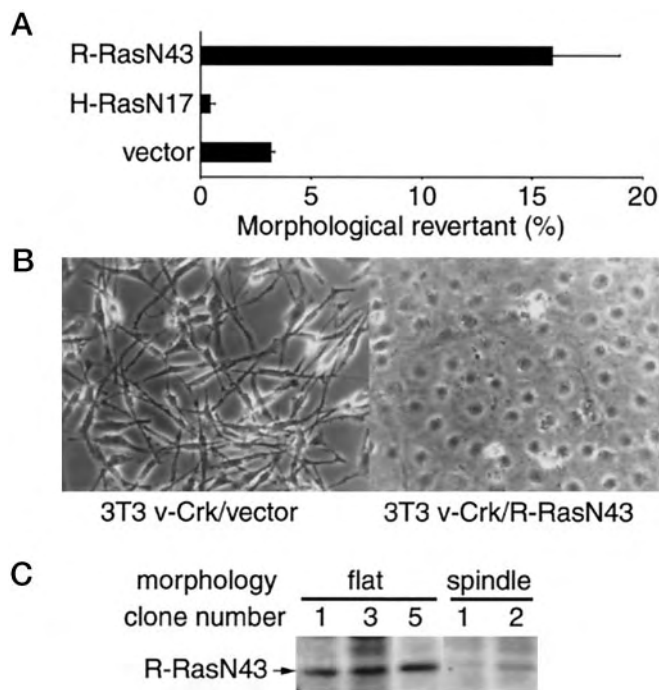
<sup>2</sup> N. Mochizuki and M. Matsuda, unpublished results.



**FIG. 4. Inability of JNK activation by GEFs specific for Rap1.** A, 293T cells were transfected with the expression vectors for the GEFs indicated at the top. JNK activities were measured by *in vitro* kinase assay with GST-c-Jun(1–79) used as a substrate. -Fold activation compared with the control is indicated at the bottom of the first column. B, total lysates were analyzed by immunoblotting with anti-C3G, anti-RasGRF, anti-Sos, or anti-FLAG antibody. Arrows indicate Epac/cAMP-GEFI, CalDAG-GEFI, and CalDAG-GEFII. C, 293T cells were transfected with the expression vectors for the GEFs indicated at the bottom, and c-Jun transcriptional activity was measured as described in the text. Bars indicate S.D.

gether, JNK activation by integrin stimulation appears to utilize, at least in part, Crk, C3G, and R-Ras.

It should be noted that another Crk-binding protein, DOCK180, also activates JNK via Rac (26). A previous report that a catalytically inactive C3G mutant inhibits JNK activation by v-Crk does not rule out the involvement of DOCK180 in Crk-dependent JNK activation, because the dominant-negative C3G mutant used in that study blocked the Crk SH3 domain, to which both C3G and DOCK180 bind (4). In this study, we have shown that dominant-negative R-Ras also inhibits v-Crk-dependent JNK activation. Dominant-negative Ras family proteins including R-Ras<sup>Asn-43</sup> exert their effects by sequestering GEFs (39–41); therefore, this result suggests that a GEF for R-Ras, probably C3G, also activates JNK activation in 293T and NIH 3T3 cells. Because both Rac<sup>Asn-17</sup> and R-Ras<sup>Asn-43</sup> mutants inhibited v-Crk-dependent activation of JNK, there seems to be at least two pathways leading to JNK activation from v-Crk. The effect of these dominant-negative mutants were partial in 293T cells and almost complete in NIH 3T3



**FIG. 5. Morphological reversion of v-Crk-NIH 3T3 cells by expression of the dominant-negative R-Ras mutant.** A, v-Crk-NIH 3T3 cells were transfected with control vector (pCAGGS-Hyg), pCAGGS-Hyg-Flag-R-Ras<sup>Asn-43</sup>, or pCAGGS-Hyg-Flag-H-Ras<sup>Asn-17</sup>. Transfectants were selected in medium containing hygromycin B for 10 days. Flat colonies showing contact inhibition were scored as revertants. B, morphology of a representative flat colony that appeared after transfection with pCAGGS-Hyg-Flag-R-Ras<sup>Asn-43</sup> (right) is compared with that of a transformed colony transfected with the control vector (left). C, representative flat revertants and spindle non-revertants appeared after transfection with pCAGGS-Hyg-Flag-R-Ras<sup>Asn-43</sup> were isolated and subjected to immunoblotting analysis with anti-FLAG antibody.

cells. This observation suggests that, in cells expressing lower amount of v-Crk such as NIH 3T3, cooperation between the C3G-R-Ras pathway and the DOCK180-Rac pathway is required for the optimal JNK activation. Moreover, it has been shown that Crk binds to and activates hematopoietic progenitor kinase 1, which stimulates JNK in a manner dependent on TAK1 and MEKK1 (42). Although the expression of hematopoietic progenitor kinase 1 is limited to hematopoietic cells, it should be considered that Crk has multiple downstream effector molecules for the activation of JNK in various cells.

Currently it is unknown why R-Ras does not activate JNK in COS cells. Similarly to R-Ras, Rho induces activation of JNK in 293T cells but not in COS cells (43). A kinase that functions in a tissue-specific manner is postulated to transduce a signal from Rho to JNK. We showed that MLK3 was required for JNK activation by R-Ras. MLK3 is expressed in most tissues and is required for JNK activation by Rac and Cdc42 in COS cells (44); therefore, another protein that connects R-Ras to MLK3 seems to function in a cell type-specific manner.

Several proteins including PI 3-kinase, raf, and RalGDS are known to bind to both H-Ras and R-Ras. Among these, PI 3-kinase is a common effector protein between H-Ras and R-Ras (15). We tested whether active PI 3-kinases stimulate JNK in 293T cells by the use of two types of active p110 subunit of PI-3 kinase. 110-CAAX is fused to the CAAX box of H-Ras (15) and pBD-110 is activated by the fusion to p85 subunit. Neither of the mutants activated JNK in 293T cells.<sup>2</sup> Thus, PI-3 kinase has been excluded from the candidate protein that activates JNK upon R-Ras activation. As far as we know, the other effector proteins of R-Ras do not active JNK either.

We have demonstrated that the dominant-negative R-Ras mutant reverts *v-crk*-dependent morphological transformation of NIH 3T3 cells. This result strongly suggests that a GEF for R-Ras, probably C3G, is required for the transformation by v-Crk. Because Rap1 does not transform NIH 3T3 cells, R-Ras appears to transduce oncogenic signal of v-Crk in NIH 3T3 cells. However, in Swiss 3T3 cells, expression of active Rap1 induces DNA synthesis (45) and oncogenic transformation (8); therefore, it remains possible that Rap1 also transduces oncogenic signals from v-Crk in a different milieu.

In conclusion, we have shown that R-Ras functions downstream to v-Crk and C3G and upstream to MLK for the activation of JNK. This pathway plays a critical role in the transformation of NIH 3T3 cells by the v-Crk oncogene product.

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## REFERENCES

- Mayer, B. J., Hamaguchi, M., and Hanafusa, H. (1988) *Nature* **332**, 272–275
- Kiyokawa, E., Mochizuki, N., Kurata, T., and Matsuda, M. (1997) *Crit. Rev. Oncog.* **8**, 329–342
- Tanaka, S., Morishita, T., Hashimoto, Y., Hattori, S., Nakamura, S., Takenawa, T., Matuoka, K., Shibuya, M., Kurata, T., Nagashima, K., and Matsuda, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 3443–3447
- Tanaka, S., Ouchi, T., and Hanafusa, H. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 2356–2361
- Gotoh, T., Hattori, S., Nakamura, S., Kitayama, H., Noda, M., Takai, Y., Kaibuchi, K., Matsui, H., Hatase, O., Takahashi, H., Kurata, T., and Matsuda, M. (1995) *Mol. Cell. Biol.* **15**, 6746–6753
- Gotoh, T., Niino, Y., Tokuda, M., Hatase, O., Nakamura, S., Matsuda, M., and Hattori, S. (1997) *J. Biol. Chem.* **272**, 18602–18607
- Bos, J. L. (1998) *EMBO J.* **17**, 6776–6782
- Altschuler, D. L., and Ribeiro-Neto, F. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 7475–7479
- Lowe, D. G., Capon, D. J., Delwart, E., Sakaguchi, A. Y., Naylor, S. L., and Goeddel, D. V. (1987) *Cell* **48**, 137–146
- Huff, S. Y., Quilliam, L. A., Cox, A. D., and Der, C. J. (1997) *Oncogene* **14**, 133–143
- Cox, A. D., Brtva, T. R., Lowe, D. G., and Der, C. J. (1994) *Oncogene* **9**, 3281–3288
- Saez, R., Chan, A. M., Miki, T., and Aaronson, S. A. (1994) *Oncogene* **9**, 2977–2982
- Lowe, D. G., Ricketts, M., Levinson, A. D., and Goeddel, D. V. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 1015–1019
- Webb, C. P., Van, A. L., Wigler, M. H., and Woude, G. F. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 8773–8778
- Marte, B. M., Rodriguez-Viciana, P., Wennstrom, S., Warne, P. H., and Downward, J. (1997) *Curr. Biol.* **7**, 63–70
- Kitayama, H., Sugimoto, Y., Matsuzaki, T., Ikawa, Y., and Noda, M. (1989) *Cell* **56**, 77–84
- Rey, I., Taylorharris, P., Vanerp, H., and Hall, A. (1994) *Oncogene* **9**, 685–692
- Niwa, H., Yamamura, K., and Miyazaki, J. (1991) *Gene (Amst.)* **108**, 193–200
- Tanaka, M., Lu, W., Gupta, R., and Mayer, B. J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 4493–4498
- Ichiba, T., Kuraishi, Y., Sakai, O., Nagata, S., Groffen, J., Kurata, T., Hattori, S., and Matsuda, M. (1997) *J. Biol. Chem.* **272**, 22215–22220
- Kawasaki, H., Springett, G. M., Toki, S., Canales, J. J., Harlan, P., Blumenstiel, J. P., Chen, E. J., Bany, I. A., Mochizuki, N., Ashbacher, A., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 13278–13283
- Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) *Science* **282**, 2275–2279
- Matsuda, M., Tanaka, S., Nagata, S., Kojima, A., Kurata, T., and Shibuya, M. (1992) *Mol. Cell. Biol.* **12**, 3482–3489
- Coso, O. A., Chiariello, R., Kalinec, G., Kyriakis, J. M., Woodgett, J., and Gutkind, J. S. (1995) *J. Biol. Chem.* **270**, 5620–5624
- van den Berghe, N., Cool, R. H., Horn, G., and Wittinghofer, A. (1997) *Oncogene* **15**, 845–850
- Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T., and Matsuda, M. (1998) *Genes Dev.* **12**, 3331–3336
- Tanaka, S., and Hanafusa, H. (1998) *J. Biol. Chem.* **273**, 1281–1284
- Nimnual, A. S., Yatsula, B. A., and Bar-Sagi, D. (1998) *Science* **279**, 560–563
- Dolfi, F., Garcia-Guzman, M., Ojaniemi, M., Nakamura, H., Matsuda, M., and Vuori, K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 15394–15399
- de Jong, R., van Wijk, A., Heisterkamp, N., and Groffen, J. (1998) *Oncogene* **17**, 2805–2810
- Ichiba, T., Hashimoto, Y., Nakaya, M., Kuraishi, Y., Tanaka, S., Kurata, T., Mochizuki, N., and Matsuda, M. (1999) *J. Biol. Chem.* **274**, 14376–14381
- Klemke, R. L., Leng, J., Molander, R., Brooks, P. C., Vuori, K., and Cheresch, D. A. (1998) *J. Cell Biol.* **140**, 961–972
- Nakashima, N., Rose, D. W., Xiao, S., Egawa, K., Martin, S. S., Haruta, T., Saltiel, A. R., and Olefsky, J. M. (1999) *J. Biol. Chem.* **274**, 3001–3008
- Khwaja, A., Lehmann, K., Marte, B. M., and Downward, J. (1998) *J. Biol. Chem.* **273**, 18793–18801
- Keely, P. J., Rusyn, E. V., Cox, A. D., and Parise, L. V. (1999) *J. Cell Biol.* **145**, 1077–1088
- Zhang, Z. H., Vuori, K., Wang, H. G., Reed, J. C., and Ruoslahti, E. (1996) *Cell* **85**, 61–69
- Ramos, J. W., Kojima, T. K., Hughes, P. E., Fenczik, C. A., and Ginsberg, M. H. (1998) *J. Biol. Chem.* **273**, 33897–33900
- Arai, A., Nosaka, Y., Kohsaka, H., Miyasaka, N., and Miura, O. (1999) *Blood* **93**, 3713–3722
- Stacey, D. W., Feig, L. A., and Gibbs, J. B. (1991) *Mol. Cell. Biol.* **11**, 4053–4064
- Powers, S., O'Neill, K., and Wigler, M. (1989) *Mol. Cell. Biol.* **9**, 390–395
- Farnsworth, C. L., and Feig, L. A. (1991) *Mol. Cell. Biol.* **11**, 4822–4829
- Ling, P., Yao, Z., Meyer, C. F., Wang, X. S., Oehrl, W., Feller, S. M., and Tan, T. H. (1999) *Mol. Cell. Biol.* **19**, 1359–1368
- Teramoto, H., Crespo, P., Coso, O. A., Igishi, T., Xu, N., and Gutkind, J. S. (1996) *J. Biol. Chem.* **271**, 25731–25734
- Teramoto, H., Coso, O. A., Miyata, H., Igishi, T., Miki, T., and Gutkind, J. S. (1996) *J. Biol. Chem.* **271**, 27225–27228
- Yoshida, Y., Kawata, M., Miura, Y., Musha, T., Sasaki, A., Kikuchi, A., and Takai, Y. (1992) *Mol. Cell. Biol.* **12**, 3407–3414