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p120 GAP Modulates Ras Activation of Jun Kinases and Transformation*

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Although recent evidence demonstrates that Ras causes transformation by activation of multiple downstream pathways, the specific role of non-Raf effector pathways is presently unknown. Although Ras causes activation of the Jun NH_2-terminal kinases (JNKs) via a Raf-independent pathway, the contribution of JNK activation to Ras transformation and the effector that mediates JNK activation have not been established. We observed that a dominant negative mutant of SEK1/JNKK, an activator of JNKs, selectively inhibited oncogenic Ras activation of JNK and Ras transformation, but not Ras activation of the p42 mitogen-activated protein kinase. In contrast, overexpression of wild type SEK1 enhanced Ras activation of JNK and transforming activity. Thus, JNK activation promotes Ras transformation. Furthermore, a dominant negative mutant of p120 GAP (designated N-GAP), a candidate Ras effector, blocked Ras, but not Raf, transformation and blocked Ras, but not Rac, activation of JNK. Since N-GAP overexpression reduced the association of p190 Rac/Rho GAP with endogenous p120 GAP, N-GAP may form nonproductive complexes with components critical for p120 GAP function. In summary, p120 GAP may function as an effector for Ras activation of JNK and Ras transformation.

Ras proteins are GDP/GTP-regulated switches that relay the signals mediated by diverse extracellular stimuli (1, 2) to activate two distinct mitogen-activated protein kinase (MAPK)[1] cascades (3, 4). Activated Ras complexes with and promotes the activation of Raf serine/threonine kinases, which then activate MAPK kinases (MEKs), which in turn activate the p42/p44 MAPKs (also called ERKs). The central role of the Raf/MEK/ERK pathway in Ras-mediated transformation is supported by the observations that kinase-deficient mutants of Raf-1, MEKs, and ERKs are potent inhibitors of Ras transformation, whereas constitutively activated mutants of Raf-1 or MEK cause tumorigenic transformation (5–11). Ras also activates a second Raf-independent kinase cascade that leads to activation of JNKs (also called SAPKs) (12–14). However, whether JNK activation contributes to Ras transformation and what effector mediates Ras activation of JNKs are presently unresolved.

Recent observations suggest that Ras may mediate its transforming actions by stimulating both Raf-dependent and Raf-independent signaling pathways. For example, there is evidence that the function of Rho family proteins is necessary for full Ras transforming activity (15–18). Furthermore, effector domain mutants of Ras, that are defective in Raf-1 activation, still retain the ability to cause tumorigenic transformation of NIH 3T3 cells, possibly via activation of Rho family proteins and JNK (19–21). Finally, the identification of non-Raf candidate Ras effectors provides additional support for the existence of Raf-independent Ras signaling pathways (22–24). These include the two Ras GTPase activating proteins (p120 and NF1 GAPs) (25–31), guanine nucleotide exchange factors for Ral proteins (32–34), and phosphatidylinositol-3-OH kinase (35). Presently, the contribution of these candidate effectors to Ras signal transduction and transformation has not been determined.

Although there is evidence that p120 GAP functions both as a negative regulator and downstream effector of Ras, the precise nature of the second role remains unresolved (25–31). The COOH terminus contains the catalytic domain that binds to Ras and promotes the hydrolysis of bound GTP. Whereas little is known about the functional role of the NH_2 terminus of p120 GAP, the presence of protein-protein interaction domains such as the Src homology 2 and 3 motifs (SH2 and SH3), as well as the pleckstrin homology domain, suggests that p120 GAP may serve as an adaptor to promote the formation of complexes with activated Ras. Consistent with this, GAP-associated proteins that interact with the SH2 or SH3 sequences have been identified (36).

Since p120 GAP may serve both a negative regulatory and a downstream effector function for Ras, experiments aimed at defining an effector function of p120 GAP based on overexpression are very difficult to interpret. Therefore, much of the information implicating p120 GAP as an effector for Ras signaling and transformation has come from studies in which NH_2-terminally truncated mutants of p120 GAP (*e.g.* N-GAP) that lack the GTPase catalytic activity have been shown to exhibit a variety of cellular activities (36). For example, we and others showed that N-GAP fragments could block Ras-mediated signaling and transformation (25, 28, 29, 31). Therefore, N-GAP may serve as a dominant inhibitory mutant of p120 GAP downstream effector function.

p120 GAP may mediate Ras signaling pathways that modulate actin cytoskeletal organization (37). Since Rho family proteins are modulators of actin organization and activate JNK (38, 39), we determined if JNK activation is important for Ras transformation and if p120 GAP may mediate Ras activation of JNK. First, we observed that dominant negative mutants of SEK1 (an activator of JNK), which selectively blocked Ras activation of JNK but not ERKs, inhibited Ras transformation.

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; ERK, extracellular signal-regulated kinase; GAP, GTPase activating protein; MEK, MAPK/ERK kinase; GST, glutathione S-transferase; HA, hemagglutinin; MBP, myelin basic protein.

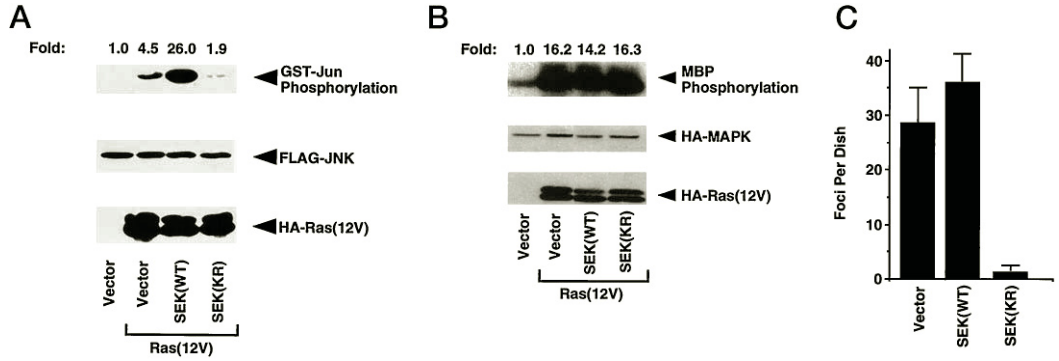

FIG. 1. **SEK1(KR) dominant negative inhibition of oncogenic Ras(12V) activation of JNK, but not ERKs, and inhibition of Ras(12V) focus-forming activity.** *A*, whereas co-expression of SEK1(WT) potentiated, SEK1(KR) blocked oncogenic Ras(12V) activation of JNK1. *B*, co-expression of SEK1(KR) did not inhibit Ras activation of p42 MAPK in transiently transfected COS-7 cells. *C*, co-expression of the mutant SEK1(KR), but not wild type SEK1, blocked oncogenic Ras(12V) focus formation in transfected NIH 3T3 cells. Similar results were obtained in three separate experiments.

Second, we found that catalytically inactive fragments of p120 GAP blocked Ras, but not Rac1, activation of JNK, and blocked Ras, but not Raf, transformation of NIH 3T3 cells. We suggest that p120 GAP may mediate Ras activation of JNK and that JNK activation contributes to Ras transformation.

EXPERIMENTAL PROCEDURES

Molecular Constructs—Expression vectors encoding wild type and kinase-deficient mutants of human SEK1 were provided by M. Karin and D. Templeton. pZIP-NeoSV(x)1 retrovirus expression vector constructs encoding NH_2-terminal fragments of human p120 GAP were described previously (28). N-GAP contains p120 GAP residues 1 to 666, whereas N-GAP/CAA*X* represents N-GAP sequences together with addition of the COOH-terminal 18-amino acid plasma membrane-targeting sequences from human Ki-Ras4B (28). pCGN-hyg expression vector constructs of N-GAP and N-GAP/CAA*X* were also generated, where expression of NH_2-terminal hemagglutinin (HA) epitope-tagged N-GAP proteins were expressed off the cytomegalovirus promoter. Expression vectors encoding FLAG epitope-tagged JNK, HA epitope-tagged p42 MAPK/ERK2, and HA-tagged Ha-Ras(12V) (pDCR) were described previously and provided by M. Karin, M. Weber, and M. White, respectively (19, 40). pZIP-*rac*1(61L), pUC-Ha-*ras*(12V) and pZIP-*raf*22W encode transforming mutants of human Rac1, Ha-Ras and Raf-1, respectively, and have been described previously (20, 41). pGEX-*jun* encodes a glutathione *S*-transferase (GST)-Jun-(1–79) fusion protein (provided by M. Karin) (42).

Cell Culture and Transformation Analyses—NIH 3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, and plasmid DNAs were introduced by calcium phosphate precipitation by procedures described previously (43). To determine the role of JNK activation in Ras transformation, cultures of NIH 3T3 cells were co-transfected with plasmid constructs encoding oncogenic Ras(12V) (10 ng/dish), together with either empty vector or vectors encoding wild type or mutant SEK1 (0.5 μg/dish). Kinase-deficient mutants of SEK1 contain mutations in the ATP binding site (Lys → Arg or Ala) and have been shown to selectively inhibit JNK, but not ERK, activation by anisomycin, Abl, Rac, or ceramide (44–47). To determine the role of p120 GAP in Ras and Raf transformation, NIH 3T3 cells were co-transfected with 25 ng of pUC-H-*ras*(12V) or 50 ng of activated Raf-1 (pZIP-*raf*22W) and 2 μg of pZIP encoding N-GAP or N-GAP/CAA*X*. All transfections were performed in triplicate, and the appearance of transformed foci was quantitated after 14 to 16 days.

JNK and MAPK Kinase Assays—To determine the ability of the wild type or mutant SEK1 to modulate oncogenic Ras activation of JNK or MAPK, COS-7 cells were transfected with 2 μg of FLAG epitope-tagged JNK or hemagglutinin (HA) epitope-tagged p42 MAPK/ERK2, 2 μg of pDCR Ha-*ras*(12V) (HA-tagged), and 2 μg of empty vector, or vector encoding wild type or dominant negative SEK1. To assess the ability of N-GAP to modulate oncogenic Ras or Rac1 activation of JNK or p42 MAPK, COS-7 cells were transfected with 1.5 μg of HA-MAPK or FLAG epitope-tagged JNK, 1 μg of pDCR-*ras*H(12V) or 1.5 μg of pCGN-*rac*1(61L), and 3 μg of a pcDNA1 construct encoding N-GAP/CAA*X*. Forty-eight h after transfection, the cells were shifted to 0.5% serum overnight. After an additional 15 h, the cells were lysed and protein levels were equalized before immunoprecipitating JNK or MAPK/ERK from the extracts with 2 μl of anti-FLAG (Kodak Eastman) or anti-HA (Babco) antibody. The appropriate immunoprecipitate was assayed for JNK activity on GST-Jun fusion protein or MAPK activity on myelin basic protein (MBP), using procedures described previously (20) and gels quantitated by scintillation counting of excised bands. The presence of similar levels of FLAG-JNK1 or HA-MAPK protein was confirmed by Western blot analysis using anti-FLAG or -HA monoclonal antibodies, respectively.

Determination of p190 Rac/Rho GAP Association with p120 GAP—To determine the consequences of N-GAP overexpression on the degree of p190 Rac/Rho GAP association with endogenous p120 GAP, COS-7 cells were transiently transfected with pDCR-Ha-*ras*(12V) and either the pCGN-hyg empty vector or pCGN-hyg encoding N-GAP. After 48 h, the cells were lysed and cleared of N-GAP by immunoprecipitating with 3 μl of anti-HA antibody. Endogenous full-length p120 GAP was then immunoprecipitated from the cleared lysates with an anti-p120 Ras GAP monoclonal (BF48, Santa Cruz Biotech). Levels of p120 GAP and of co-precipitated p190 Rho GAP were analyzed by Western blotting (p190 antiserum provided by S. Parsons).

RESULTS AND DISCUSSION

Kinase-deficient SEK1 Selectively Impairs Oncogenic Ras Activation of JNK, but Not p42 MAPK, and Ras Transformation—Since JNK is a component of the stress-activated pathway, it has been proposed that JNK activation mediates an apoptotic, rather than growth-proliferative, response (46–48). On the other hand, a direct correlation was observed between the level of JNK activation and the transforming potency of a panel of Ras mutants (8). Furthermore, JNK is an activator of the Jun transcription factor, and Jun function is necessary for Ras transformation of fibroblast cells (49, 50). To assess the contribution of JNK activation to Ras transformation, we determined if dominant negative mutants of the JNK activator, SEK1/JNKK (13, 44, 51, 52), could block Ras transforming activity. Kinase-deficient mutants of SEK1 have been shown previously to selectively block JNK activation by a variety of stimuli (44, 45, 47). First, we wanted to determine if dominant negative SEK1 could selectively block oncogenic Ras activation of JNK, but not ERK. We observed that transient transfection analyses showed that co-expression of the kinase-deficient SEK1(KR) mutant blocked oncogenic Ras activation of JNK1 (Fig. 1*A*), whereas co-expression of SEK1(WT) greatly potentiated JNK1 activation. In contrast, SEK1(KR) did not block Ras activation of p42 MAPK/ERK2 (Fig. 1*B*). Thus, dominant negative SEK1 is not an inhibitor of the Raf/MEK/MAPK pathway. These results are concordant with those described previously, where dominant negative SEK1 selectively blocked transforming Abl activation of JNK, but not ERK (44).

Next, we determined if dominant negative SEK1 inhibition of JNK activation would alter Ras(12V) transforming activity. Whereas co-transfection of SEK1(WT) caused an enhancement

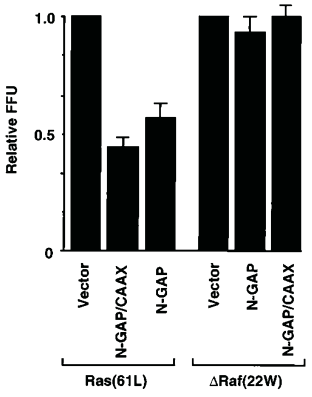

FIG. 2. **N-GAP inhibition of oncogenic Ras(12V), but not ΔRaf(22W), transforming activity.** NIH 3T3 cells were co-transfected with expression vectors encoding transforming Ha-Ras(12V) or Raf-1(22W) mutant proteins, and either with the pZIP empty vector, or with pZIP vectors encoding N-GAP or N-GAP/CAA*X* and the appearance of transformed foci scored after 14–16 days. Data shown are the average of three separate assays and are expressed as relative focus-forming units (*FFU*) normalized to the activities of oncogenic Ha-Ras(12V) or ΔRaf(22W).

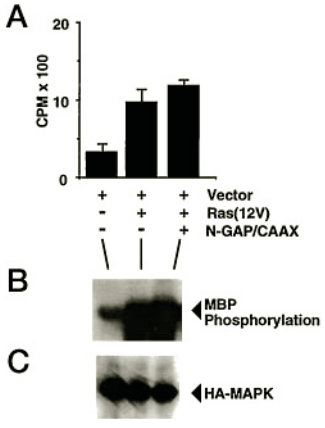

FIG. 3. **N-GAP fails to inhibit oncogenic Ras(12V) activation of p42 MAPK.** COS-7 cells were co-transfected with expression vectors encoding HA-MAPK/ERK, Ha-Ras(12V), and N-GAP/CAA*X*. Forty-eight h after transfection, the cells were harvested and used for MAPK kinase assays that were performed as described under "Experimental Procedures." Data shown are representative of three separate determinations. *A*, quantitation of phosphorylation of MBP shown in *B*; *B*, incorporation of ^{32}P into MBP; *C*, Western blot analysis using anti-HA antibody to quantitate HA-MAPK/ERK expression.

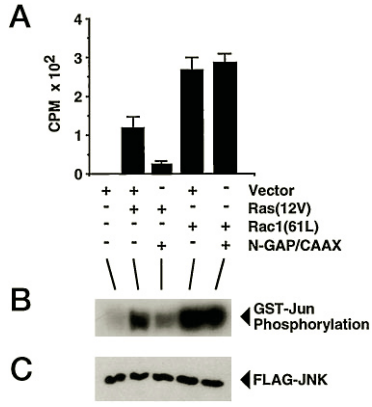

FIG. 4. **N-GAP inhibition of oncogenic Ras, but not Rac1, activation of JNK.** COS-7 cells were transfected as described above with expression vectors encoding FLAG epitope-tagged JNK, Ha-Ras(12V), Rac1(61L), and N-GAP/CAA*X* as indicated. Serum-starved cell lysates were equalized for protein concentration and then immunoprecipitated with anti-FLAG antibody (Kodak-Eastman). *A*, quantitation (above background) of the average of two separate assays for GST-Jun phosphorylation (*B*) was measured as described in Fig. 1*A*; *C*, Western blot of FLAG-JNK using anti-FLAG antibody.

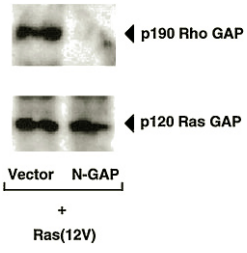

FIG. 5. **N-GAP reduction of p190 Rho GAP association with p120 GAP.** COS-7 cells were transiently transfected with pDCR-Ha-*ras*(12V) and either the pCGN-hyg empty vector or pCGN-hyg encoding N-GAP. After 48 h, the cells were lysed and cleared of N-GAP by immunoprecipitating with 3 μl of anti-HA antibody. The cleared lysates were then immunoprecipitated with an anti-p120 Ras GAP monoclonal, and the immunoprecipitates were analyzed by Western blotting for the levels of p120 GAP and co-precipitated p190 Rac/Rho GAP (p190 antiserum provided by S. Parsons).

of Ras(12V) focus-forming activity, co-transfection of SEK1 (KR) caused a very significant (80 to 90%) inhibition of Ras(12V) focus-forming activity (Fig. 1*C*). Thus, JNK activation is necessary for full Ras transformation. It has been shown previously that dominant negative mutants of Raf, MEK, and ERK also inhibit Ras transformation. Therefore, Ras activation of JNKs and ERKs are complementary, and their action together promotes Ras transformation. This contrasts with the opposing effects of ERK and JNK on apoptosis in PC12 pheochromocytoma cells (46). In these studies, apoptosis induced by nerve growth factor withdrawal was associated with increased JNK, but decreased ERK, activation. However, constitutive activation of the MEK/ERK pathway prevented apoptosis induced by epidermal growth factor withdrawal.

The importance of JNK activation, in the absence of ERK activation, for Ras transformation is suggested by our recent observation that effector domain mutants of oncogenic Ras that are impaired in activation of the Raf/MEK/ERK pathway, but still activate JNK, retain the ability to cause tumorigenic transformation of NIH 3T3 cells. Furthermore, constitutively activated mutants of Rac or Dbl family proteins (*e.g.* Dbl and Ost) are activators of JNK, but not ERK (14, 45, 53), yet cause growth transformation of NIH 3T3 cells. Thus, the consequences of JNK activation on cell behavior may be cell type-specific, causing growth (NIH 3T3), apoptosis (PC12, U937 leukemia, and others) or differentiation (12, 46–48, 54). The consequences of JNK activation are also likely to be influenced by the action of concurrent signaling events such as ERK activation (46).

N-GAP Selectively Impairs Oncogenic Ras, but Not Rac1, Activation of JNK—Ras, but not Raf, causes direct activation of JNKs (13, 14). However, the effector that mediates Ras activation of JNK has not been established. Although there is evidence that p120 GAP can serve as an effector for Ras signaling, the precise pathways that may be controlled by p120 GAP have not been established. Therefore, we next determined if p120 GAP functions as a downstream effector to mediate JNK activation via a Raf/MEK/ERK-independent pathway.

We and others showed previously that expression of NH_2-terminal fragments of p120 GAP, that lack the COOH-terminal catalytic domain (designated N-GAP), inhibited Ras-mediated signaling and transformation (25, 28, 31). Thus, N-GAP may function as a dominant negative mutant of p120 GAP. Since we found previously that a membrane-targeted version of N-GAP (designated N-GAP/CAA*X*) was slightly enhanced in this inhibitory action, we used both N-GAP and N-GAP/CAA*X* mutant

proteins for our studies. First, we determined that co-expression of N-GAP blocked oncogenic Ras(12V), but not ΔRaf(22W), focus-forming activity in NIH 3T3 transformation assays (Fig. 2). Thus, N-GAP inhibition of Ras transformation is specific and may involve antagonism of pathways distinct from those required to cause Raf transformation.

The failure of N-GAP to block Raf transformation suggested that N-GAP inhibition of Ras transformation was a consequence of inhibition of a Ras-mediated, Raf-independent pathway. Consistent with this, we found that co-expression of N-GAP did not block oncogenic Ras activation of p42 MAPK/ERK2 (Fig. 3). Thus, N-GAP inhibition of Ras transformation is not a consequence of inhibition of the Raf/MEK/ERK pathway. These observations are similar to those of Tocque and colleagues (55), who found that an anti-p120 GAP monoclonal antibody blocked oncogenic Ras-induced germinal vesicle breakdown, but not ERK activation, in *Xenopus* oocytes.

Since it has been proposed that p120 GAP may mediate Ras activation of Rho family proteins, and consequently, JNK activation, we determined if N-GAP could inhibit Ras activation of JNK. We observed that co-expression of N-GAP blocked oncogenic Ras(12V) activation of JNK. In contrast, N-GAP failed to block the ability of a constitutively activated mutant of Rac1(12V) to activate JNK (Fig. 4). Thus, p120 GAP may function as an effector to mediate Ras activation of JNK. Since Ras activation of JNK is dependent on Rac function (45), these observations suggest that p120 GAP serves to connect Ras with Rac to cause activation of JNK. However, we do not exclude the possibility that Ras can also activate JNK by other candidate effectors such as MEKK1 and phosphatidylinositol-3-OH kinase (35, 56).

N-GAP Antagonizes p190 Rac/Rho GAP Association with Endogenous p120 GAP—N-GAP contains several domains that may mediate protein-protein interactions, such as the Src homology 2 and 3 (SH2 and SH3) domains, as well as pleckstrin homology and other candidate motifs. Thus, N-GAP may inhibit Ras function by its formation of nonproductive complexes with components that are important for mediating p120 GAP downstream function. For example, the two SH2 domains are required for p120 GAP interaction with the p190 Rho/Rac GAP (57).

Since p190 functions as a Rho/Rac GAP (58), it constitutes a logical connection to promote Ras activation of a Rho family protein which then causes activation of JNKs. Therefore, we determined if co-expression of N-GAP decreased p190 association with endogenous p120 GAP. Whereas immunoprecipitation of p120 GAP from control untransfected COS-7 cells resulted in the co-precipitation of p190, the degree of p190 co-precipitation was reduced significantly in cells expressing exogenously introduced N-GAP (Fig. 5). Thus, N-GAP can antagonize the ability of proteins to associate with p120 GAP. Whether formation of a complex between Ras:p120 GAP and p190 GAP serves to inactivate its Rac/Rho GAP activity, to lead activation of Rho family proteins, remains to be determined. Whether p120 GAP association modulates p190 GAP activity, whether p190 GAP overexpression overcomes N-GAP inhibitory action, and whether an N-GAP mutant that fails to bind p190 GAP has lost the ability to block Ras transformation are experiments that may help address this question.

In summary, recent studies have shown that oncogenic Ras causes transformation by activation of Raf-dependent and Raf-independent pathways (19–21). Thus, there has been a renewed interest in defining the involvement of other Ras-binding proteins in mediating Ras signaling and transformation (22–24). Our observations implicate p120 GAP as an effector for mediating a Raf-independent signaling pathway that causes JNK activation, and that JNK activation is essential for full Ras transforming activity. These results further emphasize the complex nature of Ras signal transduction and demonstrate that Ras mediates its transforming actions through activation of multiple effector-mediated signaling pathways.

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