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 NH2-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/JNK, 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) 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 Ras 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 NH2 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 NH2-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.
Second, we found that catalytically inactive fragments of p120 GAP blocked Ras, but not Raf, activation 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 NH2-terminal fragments of human p120 GAP were described previously (28). N-GAP contains p120 GAP residues 1 to 666, whereas N-GAP/CAA 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 were also generated, where expression of NH2-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-rac1(61L), pUC-Ha-ras(12V) and pZIP-rac2(22W) encode transforming mutants of human Ras1, Ha-Ras and Raf-1, respectively, and have been described previously (20, 41). pGEX-22W encodes oncogenic Ras(12V) (10 ng/dish), together with either 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 GAP monoclonal (BF48, Santa Cruz Biotech). Levels of p120 GAP and of co-precipitated p180 RhoGAP were analyzed by Western blotting (19, 40). To determine the role of JNK activation in Ras transformation, NIH 3T3 cells were co-transfected with 25 ng of pUC-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 GAP monoclonal (BF48, Santa Cruz Biotech). Levels of p120 GAP and of co-precipitated p180 RhoGAP were analyzed by Western blotting (19, 40) 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 scanning densitometry using an 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 GAP monoclonal (BF48, Santa Cruz Biotech). Levels of p120 GAP and of co-precipitated p190 RhoGAP were analyzed by Western blotting (19, 40) 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 scanning densitometry using an 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.

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, 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. 1A), whereas co-expression of SEK1(WT) greatly potentiated JNK1 activation. In contrast, SEK1(KR) did not block Ras activation of p42 MAPK/ERK2 (Fig. 1B). 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...
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. 1C). Thus, JNK activa-
tion 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 to-
gether promotes Ras transformation. This contrasts with the
opposing effects of ERK and JNK on apoptosis in PC12 pheo-
chromocytoma 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 induc-
ted 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 conse-
quences 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—Raf—MEK—ERK—JNK axis is essen-
tial for Ras transformation. Therefore, inhibition of either
Ras or Rac may prevent Ras-induced transformation. We have
previously shown that expression of dominant negative
mutants of Rac1(61L) and Ras(12V) impaired Ras transfor-
mation completely (46). Thus, N-GAP may function at or near
the Rac—Ras12V axis. In preliminary experiments, we observed
that expression of Rac1(61L) reduced Rac—Ras12V transformation
(48). Therefore, we next determined if N-GAP may function as a
dominant negative mutant at or near the Rac—Ras—JNK axis.

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

We and others showed previously that expression of NH2-
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/CAAX) was slightly enhanced in this inhibi-
tory action, we used both N-GAP and N-GAP/CAAX mutant

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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