Oncogenic Ha-Ras-dependent Mitogen-activated Protein Kinase Activity Requires Signaling Through the Epidermal Growth Factor Receptor*

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C3H10T1/2 fibroblasts transformed by the minimal expression of oncogenic Ha-Ras (V12H10 cells) or N-Ras (K61N10 cells) have constitutive mitogen-activated protein kinase (MAPK) activity and proliferate in serum-free medium. The constitutive MAPK activity and serum-independent proliferation of V12H10 cells are sensitive to the growth factor antagonist, suramin (Hamilton, M., and Wolfman, A. (1998) Oncogene 16, 1417–1428), suggesting that Ha-Ras-mediated regulation of the MAPK cascade is dependent upon the action of an autocrine factor. Serum-free medium conditioned by V12H10 cells contains an activity that stimulates MAPK activity in quiescent fibroblasts. This MAPK stimulatory activity could be specifically blocked by the epidermal growth factor receptor (EGFR) inhibitors, PD153035 and PD158780. These inhibitors also blocked the serum-independent proliferation of V12H10 cells. Immunodepletion of conditioned medium with antibodies to transforming growth factor α and EGF significantly inhibited its ability to stimulate MAPK activity. Stable transfection of EGFR-negative NR6 and EGFR-positive Swiss3T3 cells with oncogenic (G12V)Ha-Ras demonstrated that only the Ha-Ras-transfected Swiss 3T3 cells possessed constitutive MAPK activity, and this activity was sensitive to PD153035. These data suggest that autocrine activation of the EGFR is required for the regulation of the MAPK cascade in cells minimally expressing oncogenic Ha-Ras.

The Ras GTPases are critical transducers of signals that are initiated from plasma membrane receptor tyrosine kinases and terminate in the nucleus as changes in gene expression. The c-Ras proteins are implicated in the regulation of multiple biological processes as diverse as cell proliferation, migration, and differentiation. In most instances, the expression of mutated, oncogenic forms of Ras results in cell transformation. The loss of normal growth control may result in the acquisition of anchorage-independent growth and a diminished requirement for exogenous serum or growth factors (1) and are intimately related to the ability of Ras to stimulate the MAPK cascade, a critical component of the proliferative response (2–6). Deregulated cell proliferation is thought to be one consequence of activated Ras signaling directly and persistently through the Raf kinases, upstream mediators of the MAPK cascade. Ras transformation can also be accompanied by the synthesis and secretion of various growth factors, including interleukin 1α, interleukin 6 (7), TGF-α (8–10), vascular endothelial growth factor (VEGF) (11), and heparin-binding EGF (HB-EGF) (12). The production of autocrine growth factors may therefore be a potentially significant mechanism contributing to the ability of activated Ras proteins to undermine the processes that regulate normal cell proliferation.

We recently reported that mouse fibroblasts transformed by the minimal expression of activated (G12V)Ha-Ras (V12H10 cells) were unable to proliferate in serum-free medium in the presence of suramin, a growth factor antagonist (13–15). The constitutive MAPK activity in these cells was completely abrogated by the addition of suramin. N-Ras-transformed fibroblasts (K61N10 cells) were, however, insensitive to suramin. Biochemical analysis revealed that Raf-1 failed to interact with the activated Ha-Ras protein in the V12H10 cells, but instead, Raf-1 was found to be associated with the wild-type endogenous c-N-Ras protein. Suramin treatment disrupted the c-N-Ras–Raf-1 complex in V12H10 cells but did not disrupt the N-Ras–Raf-1 complex detected in the N-Ras-transformed K61N10 cells (15). The failure of oncogenic Ha-Ras to associate with Raf-1 is contrary to current dogma, which suggests that it is the direct association of an oncogenic Ras with Raf-1 that is responsible for regulating the MAPK cascade. These observations imply that the mechanism through which Ha-Ras regulates MAPK activity was through the action of an autocrine growth factor(s) rather than through a direct association with Raf-1. We sought to determine which receptor pathway activity was required to regulate MAPK activity and the serum-independent proliferation of Ha-Ras-transformed mouse fibroblasts.

MATERIALS AND METHODS

Reagents—All reagents were molecular biology grade. [γ-32P]ATP (3000Ci/mmol) was purchased from NEN Life Science Products. Myelin basic protein (MBP) was purchased from Sigma. Mouse monoclonal antibodies against Raf-1 were purchased from Transduction Laboratories. The anti-active MAPK rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology Inc. The monoclonal antibody to TGF-α and the goat polyclonal antibodies to EGF and HB-EGF were purchased from Santa Cruz Biotechnology Inc. The rabbit polyclonal EGFR antibody, 1005, and the neutralizing mouse monoclonal EGFR antibody.
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Ab1, were purchased from Santa Cruz Biotechnology Inc. and Oncogene Science, respectively. TR10 MAPK antisera was a kind gift from M. J. Weber, University of Virginia. The EGFR inhibitor, PD153035, was purchased from Tocris Pharmaceuticals. The EGFR inhibitor, PD153035, and the broad-spectrum reversible tyrosine kinase inhibitor, PD089824, CHAPS buffer (1% w/v) CHAPS and 0.1% Nonidet P-40. Tris-buffered saline (TBS) is 150 mM sodium chloride, 50 mM Tris (pH 7.5). CHAPS lysis buffer is MOPS buffer containing 1% 4-methylphenylphosphonate, β-glycerophosphate and p-nitrophenolphosphate. CHAPS lysis buffer was made by mixing CHAPS (50 mM w/v) CHAPS and 0.1% Nonidet P-40. Tris-buffered saline (TBS) is 150 mM sodium chloride, 50 mM Tris (pH 7.5). P21 buffer is 20 mM MOPS, pH 7.4, 200 mM sucrose, 1 mM dithiothreitol, 5 mM MgCl2, and 1 mM EDTA. Cell Culture and Transfections—V12H10 and K61N10 fibroblasts are derived from normal mouse C3H10T1/2 fibroblasts by transfection with activated (G12V)Ha-Ras and (Q61K)N-Ras constructs, respectively and are described elsewhere (15). All cell lines were maintained in DMEM + fetal bovine serum. EGFR-negative NR6 cells were a kind gift from Dr. T. Kung, Case Western Reserve University, Cleveland, OH. Swiss 3T3 and NR6 fibroblasts were transfected with 100 ng of pOPRSV plasmid (Stratagene) containing activated (G12V)Ha-Ras or mock transfected using the Lipofectin method (Life Technologies, Inc.). After 48 h, cells were switched to DME + 10% fetal bovine serum containing 500 μg/ml G418. After 10–14 days, discrete foci were identified, picked, and propagated for further analysis. Cell Proliferation Assays—Cells were seeded at 2.0–5.0 × 104 cells in 100-mm culture dishes and allowed to attach overnight at 37 °C. Adherent cells were washed twice with serum-free DMEM and incubated in the presence of 1 μM PD153035 or 1 μM PD158780 in serum-free DMEM for 48 h, after which they were trypsinized and counted using a hemocytometer. Conditioned Medium—Confluent cultures of V12H10 cells were washed twice with serum-free DMEM and then incubated in serum-free DMEM for 48–72 h. The conditioned medium (CM) was collected and centrifuged at 1000 rpm for 10 min at 4 °C to remove any nonadherent cells. CM was aliquoted and stored at −20 °C. The CM was concentrated 20–30-fold using a Centriprep 3 membrane (Amicon) with a 3000-Da cut-off. Both the CM retentate and flow-through fractions were saved and stored at −20 °C. Protease Digestion—Conditioned medium was digested for 3 h at 37 °C with 10 μg/ml Streptomyces griseus Type XIV protease (Sigma), a nonspecific protease. After 3 h, fresh Type XIV protease was added, and the incubation was allowed to proceed for an additional 2 h. The protease was inactivated by heating at 100 °C for 15 min. Preparation of Cell Lysates—Cell monolayers were washed twice with ice-cold TBS, scraped, and pelleted by low speed centrifugation at 800 rpm for 5 min at 4 °C. The cell pellet was resuspended in 200–500 μl of CHAPS lysis buffer and incubated on ice for 20 min. Insoluble cellular debris was pelleted by centrifugation at 13,000 rpm at 4 °C in a microcentrifuge. The clarified lysates were then washed with TBS + Nonidet P-40 and incubated with the appropriate antibody in TBS + Nonidet P-40 containing 10% serum. The membranes were washed three times with TBS + Nonidet P-40 and incubated with the appropriate antibody in TBS + Nonidet P-40 containing 10% serum. The membranes were washed extensively with TBS + Nonidet P-40 and then incubated with horseradish peroxidase-conjugated secondary antibody in TBS + Nonidet P-40 containing 10% serum. The membranes were washed again extensively with TBS + Nonidet P-40, and immunoreactive bands were visualized by enhanced chemiluminescence (ECL). Immunoprecipitation of Ras/Raf-1 Complexes—Cell monolayers were washed twice with ice-cold TBS, scraped, and pelleted by centrifugation at 800 rpm for 5 min at 4 °C. The cell pellet was lysed in 500 μl of ice-cold CM for 30 min. Lysates were centrifuged at 13,000 rpm for 10 min at 4 °C in a microcentrifuge. The clarified lysates were pre-absorbed with 200 μl of protein A-Sepharose (PA) for 10 min on ice and centrifuged at 13,000 rpm for 5 min at 4 °C in a microcentrifuge (PA was prepared as a 1:4 slurry in TBS containing 0.1% Nonidet P-40). The protein concentration of the lysates was determined by the Bradford assay. 500 μg of cell lysates were incubated for 3 h with 50 μl of Ras antisera (15) at 4 °C with constant rotation. Insoluble material was pelleted by centrifugation at 13,000 rpm for 10 min at 4 °C. Lysates were transferred to clean Eppendorf tubes containing 400 μl of PAS and allowed to precipitate for an additional h at 4 °C. The immunoprecipitates were collected by centrifugation and washed five times with ice-cold P21 + Nonidet P-40 buffer. The supernatant, containing the immunoprecipitates, was heated for 5 min at 100 °C. Immunoprecipitates were resolved on 8% SDS-PAGE gels and immunoblotted for Raf-1. Immunoreactive bands were visualized by ECL. Immunoprecipitation of EGFR Ligands from CM—400 μl of CM was immunoprecipitated overnight at 4 °C with 10 μg of EGFR ligand antibodies (TGF-α, HB-EGF, and EGF) control antibodies precoated to Protein G-Sepharose. The immunocomplexes were separated from the CM by centrifugation through a 0.45 μm Ultrafree-MC filter unit (Millipore), and the CM was tested for its ability to activate MAPK in quiescent fibroblasts. MAPK Activity Assay—Cells were incubated for 48 h in serum-free media in the presence or absence of the following compounds: 200 μM suramin; 1 μM PD153035; 1 μM PD158780; 5 μM PD808982; Cells were stimulated for 10 min with V12H10-conditioned medium, or PDGF-BB, FGF-2, or EGF. The cell monolayers were washed twice with ice-cold TBS, scraped, and lysed in CHAPS lysis buffer. MAPK was immunoprecipitated from equal amounts of cell lysate protein for 2 h at 4 °C with constant rotation using 0.5 μl of TR10 anti-MAPK serum precleared with 20 μg of Protein A-Sepharose. The immunoprecipitates were washed three times with P21 + Nonidet P-40 buffer and twice with P21 buffer, 50 μl of kinase buffer (P21 buffer containing 10 μM ATP, 10 mM MgCl2, 10 μM [γ-32P]ATP, and 10 μM phosphatase inhibitors) containing 25 μM Mg2+ ATP was added to each immunoprecipitate and incubated at 37 °C for 20 min. The kinase reaction was stopped by the addition of 50 μl of 2 × Laemmli buffer, and the phosphorylated proteins were resolved on a 15% SDS-PAGE gel. Phosphorylated MAB was visualized and quantitated on a Molecular Dynamics PhosphorImager.

RESULTS

Detection of a MAPK-stimulating Activity in Conditioned Medium—To determine whether Ha-Ras-transformed V12H10 cells secrete a factor that is responsible for regulating their constitutive MAPK activity, sub-confluent V12H10 cells were incubated in serum-free DMEM for 72 h. Parallel samples (72 h in serum-free DMEM) were collected from sub-confluent parental C3H10T1/2 fibroblasts. This CM was then tested for its ability to activate MAPK. C3H10T1/2 cells plated in 100-mm culture dishes and made quiescent by serum starvation for 48 h were stimulated with 5 ml of CM for 10 min at 37 °C. The cells were then scraped and lysed in 1% CHAPS buffer, and equal amounts of clarified cell lysate protein were resolved on 8% SDS-PAGE gels and processed for Western blot analysis. V12H10:CM stimulated the activation of p42 and p44 MAPK as indicated by the detection of phosphorylated p42 and p44 MAPK proteins (Fig. 1A). These observations were supported by specifically immunoprecipitating MAPK from identical cell lysates and measuring the activity of the immunoprecipitated MAPK in a direct kinase assay (data not shown). Medium conditioned by the parental C3H10T1/2 cells did not significantly activate MAPK. Initial characterization of the MAPK-stimulating activity in CM demonstrated that it could be centrifuged through a 3000-Da cut-off membrane, with all of the MAPK-stimulating activity remaining in the retentate fraction. The activity in V12H10:CM was titratable and could be diluted 30-fold with no significant effect upon its ability to activate MAPK (data not shown). Further biochemical analysis demonstrated that MAPK-stimulating activity could be absorbed to a heparin-Sepharose CL4B column (data not shown) and was heat stable (100 °C for 10 min; data not shown)). Polypeptide growth factors and stimulatory lipids such as lysophosphatidic acid (16, 17) can both activate the MAPK cascade. To determine whether the MAPK-stimulating activity in the V12H10:CM was protein in nature, CM was digested for 5 h at 37 °C with S. griseus Type XIV protease followed by inactivation of the protease by heat denaturation. The ability of
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the protease-digested CM to stimulate MAPK was tested by challenging quiescent C3H10T1/2 cells with either naive DMEM (fresh serum-free DMEM) or protease-digested CM for 10 min at 37 °C. The effect of prolonged incubation of the CM at 37 °C upon its ability to activate MAPK was determined by incubating CM for 5 h with heat-denatured Type XIV protease. Cells were stimulated with control and protease-treated CM, lysates were prepared, and MAPK was immunoprecipitated. The activity of the immunoprecipitated MAPK was measured in a direct kinase assay using MBP as a MAPK substrate (18). Fig. 1B shows that CM digested with Type XIV protease (CM + Pr) fails to stimulate MAPK activity, being no better at stimulating MAPK than the naive DMEM control. However, the CM incubated for 5 h with denatured protease (CM + dnPr) still possessed significant, though reduced, activity. The observation that prolonged incubation of the CM at 37 °C had a diminished ability to activate MAPK might indicate that it contains more than one polypeptide growth factor, of which one or more might be sensitive to prolonged incubation at 37 °C. However, we conclude that the MAPK-stimulating activity in V12H10:CM possesses many of the biochemical properties of polypeptide growth factors.

CM Contains EGFR Ligand(s)—The production of several growth factors including members of the EGF family has been associated with the expression of oncogenic Ras and can be implicated in the autocrine regulation of cell growth (8–10, 12). Gangarosa et al. (20) demonstrated that the mRNA transcripts of the EGFR ligands, TGF-α, HB-EGF, and amphiregulin were induced in epithelial cells transformed by activated Ha-Ras and Ki-Ras and that TGF-α protein was secreted. To investigate whether EGFR ligands may be responsible for the MAPK-stimulating activity in V12H10:CM, 400 µl of V12H10:CM was incubated overnight with antibodies to mouse EGF, HB-EGF, and TGF-α or with a control antibody. The immunocomplexes were separated from the CM by low speed centrifugation through a 0.45-µm filter. The CM was then assayed for its ability to stimulate MAPK activity by the addition of 100 µl of the immunodepleted CM to quiescent fibroblasts in 1.0 ml of serum-free medium (final concentration of CM was 0.1 ×). Cells were stimulated for 10 min at 37 °C, then scraped and lysed. MAPK was immunoprecipitated, and its activity was measured using MBP as a substrate in a direct kinase assay. CM incubated overnight either alone or with a control antibody strongly activated MAPK to an equal degree. In contrast, CM immunoprecipitated with antibodies to either EGF or TGF-α possessed a significantly reduced ability to stimulate MAPK, being slightly better than the naive DMEM control at activating MAPK. In contrast, the antibody to HB-EGF had no effect on the ability of the CM to stimulate MAPK activity (Fig. 1C). Although these observations strongly support the conclusion that EGF and TGF-α are stimulatory factors present in the V12H10:CM, it does not exclude the possibility that additional polypeptide growth factors, including other members of the EGF family, may also be present in the CM.

To determine whether the EGFR ligands present in V12H10:CM were solely responsible for stimulating MAPK activity in quiescent cells, specific pharmacological inhibitors of the EGFR were tested for their ability to block MAPK activation in response to stimulation with V12H10:CM. Confluent C3H10T1/2 cells were incubated for 48 h in 5 ml of serum-free DMEM. In parallel cultures, cells were incubated for 48 h in serum-free medium containing two highly specific inhibitors of the EGFR, PD153035 (19, 20) and the related compound, PD158780 (1 µM each inhibitor). Both PD153035 and PD158780 are competitive inhibitors of ATP binding, effectively blocking receptor tyrosine kinase activity. Cells were

Fig. 1. A, activation of MAPK by serum-free CM from V12H10 or C3H10T1/2 fibroblasts. Quiescent C3H10T1/2 fibroblasts were stimulated for 10 min by the addition of serum-free CM from V12H10 or control C3H10T1/2 fibroblasts. Cell lysates were prepared, and 200 µg of lystate was resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and immunoblotted with an antibody that specifically recognizes active, phosphorylated p42 and p44 MAPKs. The data is representative of three independent experiments, std, standard. B, the MAPK-stimulating activity in V12H10:CM is protease-sensitive. V12H10:CM was incubated with 10 µg/ml native or heat-denatured S. griseus Type XIV protease (Sigma), a nonspecific protease, for 5 h at 37 °C. Quiescent C3H10T1/2 cells were challenged with naive DMEM, CM, CM incubated with protease (CM + Pr), or with heat-inactivated protease (CM + dnPr) for 10 min. Cell lysates were prepared, and MAPK was immunoprecipitated. MAPK activity was measured in a direct kinase assay using MBP as a substrate. The data is representative of two independent experiments. C, the MAPK-stimulating activity in V12H10:CM can be immunodepleted with antibodies to EGFR ligands. 400 µl of V12H10:CM was immunoprecipitated overnight at 4 °C with antibodies to the EGFR ligands, TGF-α, EGF, and HB-EGF (10 µg antibody per immunoprecipitation) or with 10 µg of nonimmune mouse immunoglobulin. Naive DMEM and V12H10:CM were incubated overnight at 4 °C in the absence of antibody as controls. Quiescent C3H10T1/2 cells were challenged for 10 min with 100 µl of the immunodepleted CM by its direct addition to cells in 1 ml of serum-free DMEM. Cell lysates were prepared, and MAPK was immunoprecipitated. MAPK activity was measured in a direct kinase assay using MBP as a substrate. The data is representative of two independent experiments. IP, immunoprecipitating antibody; NI, nonimmune mouse immunoglobulin.
then stimulated with naive DMEM, 20 ng/ml EGF, or with 1 ml of V12H10:CM for 10 min. Cell lysates were prepared, MAPK was immunoprecipitated, and its activity was measured in a direct kinase assay. After 48 h in serum-free medium, control C3H10T1/2 cells contained negligible MAPK activity. EGF and CM strongly activated MAPK in control, untreated cells. Naive DMEM did not activate MAPK. In cell cultures treated with the EGFR antagonists, both PD153035 and PD158780 completely blocked the ability of EGF and V12H10:CM to activate MAPK (Fig. 2A), indicating that CM stimulates the MAPK cascade through its ability to activate EGFR tyrrosine kinase activity.

Further evidence implicating a critical role for the EGFR in regulating MAPK activity in response to CM stimulation was obtained using the EGFR-negative Swiss 3T3 cell line, NR6, which does not respond to EGF stimulation (21, 22). Quiescent Swiss 3T3 and NR6 fibroblasts stimulated with either CM or EGF were assayed for factor-dependent MAPK activation. Both CM and EGF activated MAPK in EGFR-positive Swiss 3T3 cells but failed to activate MAPK in EGFR-negative NR6 cells (Fig. 2B). Many RTKs bind and/or activate common intermediates and signaling molecules. Receptor activation results in autophosphorylation of the receptor that creates specific docking sites for the SH2 domains of several well characterized signaling molecules, including the recruitment of the RasGEF complex of She-Grb2-Sos (23–25) and the binding of phosphati-
didylinositol 3-kinase, Src, Shp, and phospholipase Cγ (reviewed in Refs. 26 and 27). To eliminate the possibility that there was a global defect in the RTK signaling cascade, resulting in the failure of the CM to activate MAPK in NR6 cultures, NR6 cells were stimulated with either 10 ng/ml PDGF BB or 5 ng/ml FGF2 for 10 min and examined for MAPK activation. Both PDGF and FGF2 treatment resulted in MAPK activity similar to that observed with Swiss 3T3 cells, indicating that the mechanism(s) regulating RTK-dependent activation of the MAPK cascade were intact.

The possibility remained that EGFR activation was medi-
ated through unknown nonligand-dependent mechanisms. To this end, we tested the ability of V12H10:CM to stimulate MAPK activity in the presence of an antibody that blocks the ligand binding domain of the human EGFR (Ab1; Oncogene Science). We have previously observed that both mouse EGF and V12H10:CM are able to stimulate MAPK in human foreskin fibroblasts. Therefore, 2 x 10^5 human foreskin fibroblasts in 6-well plates were made quiescent by serum starvation for 48 h and incubated overnight with an excess (2.5 μg/well) of Ab1 or a nonneutralizing EGFR antibody (1005; Santa Cruz Biotechnology). Cells were stimulated with EGF or CM for 10 min at 37 °C. Cell lysates were prepared, and MAPK activity was measured as previously described. Fig. 2C demonstrates that the neutralizing EGFR antibody, Ab1, blocks MAPK activation in response to 0.2–1 ng/ml EGF and severely abrogates MAPK activation in response to stimulation with 2.5 ng/ml EGF. Ab1 almost completely blocked all MAPK activation in response to stimulation with CM, producing a weak activation similar to that observed using control naive DMEM. In contrast, the nonneutralizing control EGFR antibody had no effect. This data suggests that CM stimulates MAPK activation through the activation of the EGFR in a ligand-dependent manner.

We can therefore conclude from the data presented in Figs. 1A and 2C that the CM prepared from V12H10 cells contains EGFR ligands that stimulate MAPK activity in quiescent cells in a receptor-ligand-dependent manner and that the EGFR and its tyrrosine kinase activity is required to activate the MAPK cascade. Furthermore, the ability of the CM to stimulate MAPK is a function of the EGFR ligands present in the CM alone.

Role of the EGF Receptor in the Proliferation of V12H10 Cells—Our earlier studies using Ras-transformed cells indi-
cated that the serum-independent proliferation of V12H10 cells was sensitive to the actions of suramin (15), a nonspecific growth factor antagonist (13, 14). This observation implied that the serum-independent proliferation of V12H10 cells was regulated by an autocrine growth factor(s). Because EGFR ligands are present in V12H10-CM, we sought to determine the importance of the EGFR in the regulation of both the constitutive MAPK activity and the serum-independent proliferation of V12H10 cells. Both Ha-Ras-transformed V12H10 cells and N-Ras-transformed K61N10 cells were incubated in serum-free medium in the presence of 1 μM PD153035 and PD158780. In addition, cells were incubated with 200 μM suramin and 10 μM PD089828 (28), a broad-range RTK inhibitor. After 48 h the cells were lysed, and MAPK immunoprecipitates were assayed for activity. Both suramin and PD089828 almost completely abrogated the constitutive MAPK activity in the V12H10 cells, providing strong evidence to suggest that MAPK activity in V12H10 cells is regulated in an autocrine manner. Although the exact mechanism of suramin action is not clear, PD089828 functions as a competitive inhibitor of ATP binding to a wide range of receptors (PDGFR, EGFR, and FGFR), demonstrating that RTK activity is required for persistent MAPK signaling in Ha-Ras-transformed V12H10 cells. More specifically, both PD153035 and PD158780 abrogated the MAPK activity, indicating that the EGFR was responsible for regulating the constitutive MAPK activity in V12H10 cells (Fig. 3A). In contrast, the constitutive MAPK activity in the N-Ras-transformed K61N10 fibroblasts was completely insensitive to the actions of all the RTK inhibitors. This data was similar to our previous findings that the N-Ras-transformed cells were refractory to suramin (15). The failure of the inhibitors to abrogate MAPK activity in K61N10 cells supports our previous conclusions that N-Ras directly regulates the MAPK cascade through its association with Raf-1. Furthermore, PD153035 did not block the ability of V12H10 cells to respond to either PDGF BB (Fig. 3B) or FGF2 or insulin-like growth factor 1 (data not shown), confirming the specificity of the inhibitor and further implicating the EGFR as the regulator of the MAPK activity in V12H10 cells.

V12H10 and K61N10 cells were incubated in serum-free medium containing 1 μM PD153035 and PD158780 to confirm that blocking EGFR function inhibited serum-free proliferation. After 48 h in serum-free medium, both V12H10 and K61N10 cells had almost doubled in number. PD153035 and PD158780, however, completely blocked the serum-independent proliferation of the V12H10 cells but not the K61N10 cells (Fig. 3C). These data provide strong evidence to support our hypothesis that the EGFR and its activity are required and necessary for the regulation of the MAPK cascade and the serum-independent proliferation of Ha-Ras-transformed V12H10 cells. Furthermore, the expression of minimal levels of activated Ha-Ras is insufficient to directly modulate MAPK activity and cell proliferation but requires EGFR activity for these biological events to occur. The minimal expression of activated N-Ras is, in contrast, sufficient to directly promote proliferation and activation of the MAPK cascade.

**PD153035 Disrupts the c-N-Ras-Raf-1 Complex in V12H10 Cells**—We have previously demonstrated that Ha-Ras fails to associate with c-Raf-1 in Ha-Ras-transformed V12H10 cells. Raf-1 was found associated with endogenous c-N-Ras, with this complex being disrupted by pretreatment with suramin. The disruption of this Ras-Raf complex correlated with the complete abrogation of constitutive MAPK activity and the inhibition of serum-independent proliferation (15). We, therefore, sought to determine whether the inhibition of EGFR activity by PD153035 disrupted the c-N-Ras-Raf-1-signaling complex.

![Fig. 3](image-url)

**Fig. 3.** A, EGFR inhibitors block the constitutive MAPK activity in Ha-Ras-transformed V12H10 cells. Ha-Ras-transformed V12H10 cells and N-Ras-transformed K61N10 cells were incubated for 48 h in serum-free DMEM in the presence or absence of 1 μM PD153035 or PD158780, 200 μM suramin or 10 μM PD089828. Cell lysates were then prepared, and MAPK was immunoprecipitated and its activity toward MBP measured in a direct kinase assay. The data is representative of three independent experiments. B, PD153035 does not inhibit MAPK activation by PDGF. V12H10 cells were incubated for 48 h in the absence or presence of 1 μM PD153035 and then stimulated with 5 ng/ml PDGF BB for 10 min. Cell lysates were then prepared, and MAPK was immunoprecipitated and its activity toward MBP measured in a direct kinase assay. The data is representative of three independent experiments. C, EGFR inhibitors block the serum-independent proliferation of V12H10 cells. Ha-Ras-transformed V12H10 and N-Ras-transformed K61N10 cells were incubated for 48 h in serum-free DMEM containing 1 μM PD153035 or PD158780. After 48 h, the cells were trypsinized and counted using a hemocytometer. Results are expressed as mean ± S.E. Values for V12H10 and K61N10 cells were incubated for 48 h in serum-free DMEM in the presence or absence of 1 μM PD153035. Ras proteins were immunoprecipitated from both cell lines, and the
immunoprecipitates were resolved by SDS-PAGE gels, transferred to a polyvinylidene difluoride membrane, and immunoblotted for Raf-1. Incubation of V12H10 cells with PD153035 significantly reduced the amount of Raf-1 associated with c-N-Ras. Complexes of Raf-1 with N-Ras in K61N10 cells were refractory to the EGFR inhibitor (Fig. 4). No Raf-1 was found to coimmunoprecipitate with Ha-Ras even though both the N-Ras and Ha-Ras antisera immunoprecipitate similar amounts of Ras protein (Ref. 15 and data not shown). This observation was similar to our previous findings in V12H10 and K61N10 cells treated for 48 h with suramin and correlated with the PD153035-mediated inhibition of MAPK activity. The data suggests that inhibition of EGFR function by either suramin or, more specifically, PD153035 disrupts the stable association of c-N-Ras with Raf-1 in Ha-Ras-transformed V12H10 cells. The disruption of this c-N-Ras-Raf-1 complex correlates with the abrogation of MAPK activity in V12H10 cells and the inhibition of serum-independent proliferation.

**FIG. 4.** PD153035 destabilizes the N-Ras-Raf-1-signaling complex in V12H10 cells. V12H10 and K61N10 cells were incubated for 48 h in serum-free DMEM in the presence or absence of 1 μM PD153035. Ras was immunoprecipitated using Ras isofrom-specific antisera (15), and the immunoprecipititates (IP) were resolved on 6% SDS-PAGE gels, transferred to a polyvinylidene difluoride membrane, and immunoblotted for Raf-1. Immunoreactive bands were visualized by ECL. Nt, nonimmune serum; Ht, Ha-Ras antisera; N, N-Ras antiserum. The Ha-Ras and the N-Ras antisera immunoprecipitate similar amounts of Ras proteins (Ref. 15 and data not shown). The data is representative of two independent experiments.

(G12V)Ha-Ras-dependent Activation of MAPK Is Downstream of the EGFR—Both EGFR-positive Swiss3T3 and EGFR-negative NR6 fibroblasts were stably transfected with activated (G12V)Ha-Ras to further examine the role of the EGFR in regulating MAPK activity in cells minimally expressing activated Ha-Ras. Ten Ha-Ras-transfected NR6 clones and six Ha-Ras-transfected Swiss 3T3 clones were incubated in serum-free medium for 48 h, and the MAPK activity in cell lysates was measured using MBP as a substrate. Transfection of the EGFR-negative NR6 cells with activated Ha-Ras did not stimulate MAPK activity in any of the NR6 clones (e.g. NR6VH1 and NR6VH5 cells) tested (Fig. 5A). EGF still failed to activate MAPK in these NR6 clones, whereas PDGF was a strong activator of MAPK activity, demonstrating that the RTK-dependent MAPK activation pathway was still intact. In contrast, the Ha-Ras-transfected Swiss 3T3 cells (S3VH1, S3VH2, S3VH3, and S3VHPC cells) had elevated MAPK activity, ranging from 1.35- to 4.24-fold above the basal MAPK activity in parental Swiss 3T3 cells. Representative data from several clones is shown in both Figs. 5A and B. Ha-Ras-transfected Swiss 3T3 cells were incubated in serum-free DMEM with or without 1 μM PD153035 for 48 h to examine the role of the EGFR in the elevation of MAPK activity. PD153035 abrogated the constitutive MAPK activity in each of the Ha-Ras-transfected Swiss 3T3 clones, reducing the MAPK activity to the basal level measured in serum-starved parental Swiss 3T3 cells (Fig. 5B). These data suggest that EGFR activity appears to be directly involved in the maintenance of the elevated, constitutive MAPK activity in cells minimally expressing activated Ha-Ras. Furthermore, MAPK activity is downstream of the EGFR in cells expressing activated Ha-Ras.

**FIG. 5.** A, activated Ha-Ras does not induce MAPK activity in EGFR-negative NR6 cells. EGFR-negative NR6 and EGFR-positive Swiss 3T3 cells were transfected with (G12V)Ha-Ras as described. G418-resistant clones were isolated and propagated for further analysis. Constitutive MAPK activity was detected by incubating clonal cell cultures in serum-free DMEM for 48 h. Cell lysates were prepared, and MAPK was immunoprecipitated and its activity measured using MBP as a substrate. S3VH1, S3VH2, S3VH3 are independent Ha-Ras-transfected Swiss 3T3 clones. S3VHPC is a mixed population of at least 40 individual Ha-Ras-transfected Swiss 3T3 clones. NR6VH1 and NR6VH5 are independent and representative Ha-Ras-transfected NR6 clones. The data is representative of three independent experiments. B, PD153035 inhibits MAPK activity in Ha-Ras-transfected Swiss 3T3 cells. The Ha-Ras-transfected Swiss 3T3 cell clones, S3VH1, S3VH2, S3VH3, and S3VHPC, were incubated for 48 h in serum-free DMEM with or without 1.0 μM PD153035. Cell lysates were prepared, and MAPK was immunoprecipitated and its activity measured using MBP as a substrate. The data is representative of three independent experiments.

**DISCUSSION**

The expression of activated Ras proteins is transforming as measured by alterations in cell morphology, the ability to grow in soft agar, constitutive MAPK activity, and a diminished requirement for exogenous growth factors or serum. The mechanisms by which activated Ras proteins mediate their effects are believed to be through their direct interaction with downstream effectors, resulting in the persistent activation of Ras-dependent signaling pathways. We have previously demonstrated, however, under conditions of minimal expression, that activated Ha-Ras does not interact with Raf family members (15). Regulatory kinases of the MAPK cascade (4, 29, 30). In V12H10 cells, constitutive MAPK activity depends on an autocrine factor, which functions through c-N-Ras to activate MAPK. Analysis of serum-free medium conditioned by V12H10 cells detected an activity that was capable of stimulating
MAPK. This activity was titratable, could be concentrated, and was heat-stable but protease-sensitive, suggesting that the activity was protein in nature. The ability of the activity to stimulate MAPK could be blocked by (i) immunodepleting it of the EGFR ligands, TGF-α and EGF, (ii) blocking the EGFR-ligand domain with a neutralizing antibody, and (iii) specific pharmacological inhibitors of the EGFR. The CM activity was able to compete with 125I-EGF for specific binding to the EGFR of A431 cells (data not shown). CM also failed to stimulate MAPK activity in EGFR-negative fibroblasts, further indicating that the activity in V12H10:CM was an EGFR ligand(s). Furthermore, inhibition of the EGFR was sufficient to block the serum-independent proliferation of Ha-Ras-transformed V12H10 cells, demonstrating that the EGFR and its activity is required for this characteristic of Ha-Ras transformation.

Previously, it was demonstrated that activated Ha-Ras or Ki-Ras, but not activated Raf-1, was sufficient to transform rat intestinal epithelial (RIE) cells (10, 20). The addition of exogenous TGF-α to parental RIE cells was sufficient to induce the morphological aspects of Ras-transformed RIE cells. This data demonstrated that Raf activity alone was not sufficient to transform these cells and that additional Ras-dependent signaling events must work in concert with Raf-1 activation to induce full-cell transformation. This is in contrast to mouse fibroblasts, which are readily transformed by activated Raf-1 (31–33). The observation in RIE cells has analogy with Ras mutants, which fail to either bind Raf-1 or stimulate membrane ruffling. These two mutant Ras proteins are, individually, nontransforming yet can cooperate to induce cell transformation (34, 35), supporting the hypothesis that the activation of at least two Ras-dependent signaling events is required for Ras transformation. The ability of exogenous TGF-α to mimic the morphological changes of Ras-transformed RIE cells suggested that the EGFR is involved in certain limited aspects of the transformed cell phenotype.

We have extended these observations to demonstrate that minimally expressed, oncogenic Ha-Ras protein requires the cooperative nature of an autocrine factor to produce a fully transformed phenotype. The observation that the EGFR is involved in the process of Ha-Ras-mediated RIE cell transformation can now be extended to include mouse fibroblasts. Like Gangarosa et al., (20), Ha-Ras-mediated cell transformation of C3H10T1/2 fibroblasts requires the activity of the EGFR and is modulated by the autocrine production of an EGFR ligand(s). In contrast though, oncogenic N-Ras is sufficient to transform mouse fibroblasts independently of EGFR activity and is most likely a function of the stable association of N-Ras with Raf-1 in the N-Ras-transformed cells. It has yet to be determined whether N-Ras can fully transform RIE cells independently of EGFR function. The EGFR-dependent regulation of the serum-independent proliferation of Ha-Ras-transformed V12H10 cells also requires the stable association of c-N-Ras with Raf-1, and that inhibition of EGFR activity results in the destabilization of this signaling complex. These observations would suggest that N-Ras function is immediately downstream of the EGFR and that N-Ras might be predominantly activated in response to EGFR ligand stimulation.

Fig. 6 represents a potential model of MAPK activation by minimally expressed oncogenic Ha-Ras. The presence of activated Ha-Ras through a Raf-1-independent and unidentified pathway induces the production and/or secretion of EGFR ligands that may accumulate in the culture medium as free ligand and/or associated with extracellular matrix or cell surface components. The interaction between ligand and EGFR activates the receptor, stimulating a series of signaling pathways (26, 27) including the formation of an active signaling complex between c-N-Ras and Raf-1, which stimulates the MAPK cascade and, ultimately, cell proliferation. The inclusion of EGFR inhibitors in the culture medium prevents the activation of the EGFR and the formation of the N-Ras-Raf-1-signaling complex and subsequently inhibits cell proliferation. Cells expressing an activated N-Ras, however, have persistent MAPK signaling due to the presence of a stable complex between N-Ras and Raf-1, which is refractory to growth factor antagonists.

The proposed model also suggests that, similar to the Rho GTPase cascade that regulates changes in cell shape and the generation of microspikes, filopodia, and lamellipodia (36–38), the Ras GTPases may also be organized into a GTPase cascade where, in this instance, Ha-Ras function is upstream of N-Ras function. This hypothesis can be readily integrated into the knowledge that at least two independent Ras signaling events are required for cell transformation (34, 35) and that oncogenic Ha-Ras and c-N-Ras regulate distinct signaling pathways that cooperate to induce cell transformation.

The current data suggesting that Ha-Ras directly regulates MAPK activity appears to be a consequence of the supra-physiological levels of activated Ras expression (6, 15, 18, 39). These conditions are likely to activate all Ras-dependent signaling cascades in a persistent but promiscuous manner. The use of activated Ras proteins expressed at near physiological levels certainly indicates that the c-Ras isoforms may regulate distinct signaling cascades and that mechanisms exist within cells to tightly regulate and coordinate Ras signaling. Future studies will be aimed at identifying those signaling pathways that are immediately downstream of Ha-Ras and to fully identify the mechanism by which Ha-Ras regulates N-Ras function.

Acknowledgment—We thank Crystal Weyman for her helpful discussion and critical review of the manuscript.

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