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Differential Requirement for Rho Family GTPases in an Oncogenic Insulin-like Growth Factor-I Receptor-induced Cell Transformation*

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Insulin-like growth factor I receptor (IGFR) plays an important role in cell growth and transformation. We dissected the downstream signaling pathways of an oncogenic variant of IGFR, Gag-IGFR, called NM1. Loss of function mutants of NM1, Phe-1136 and dS2, that retain kinase activity but are attenuated in their transforming ability were used to identify signaling pathways that are important for transformation of NIH 3T3 cells. MAPK, phospholipase Cγ, and Stat3 were activated to the same extent by NM1 and its two mutants, suggesting that activation of these pathways, individually or in combination, was not sufficient for NM1-induced cell transformation. The mutant dS2 has decreased IRS-1 phosphorylation levels and IRS-1-associated phosphatidylinositol 3′-kinase activity, suggesting that this impairment may be in part responsible for the defectiveness of dS2. We show that Rho family members, RhoA, Rac1, and Cdc42 are activated by NM1, and this activation, particularly RhoA and Cdc42, is attenuated in both mutants of NM1. Dominant negative mutants of Rho, Rac, and Cdc42 inhibited NM1-induced cell transformation, as measured by focus and colony forming ability. Dominant negative Rho most potently inhibited the focus forming activity, whereas Cdc42 was most effective in inhibiting the colony forming ability of NM1-expressing cells. Conversely, constitutively activated (ca) Rho is more effective than ca Rac or ca Cdc42 in rescuing the focus forming ability of the mutants. By contrast, ca Cdc42 is most effective in rescuing the colony forming ability of both mutants.

Insulin-like growth factor-I receptor (IGFR)[1] is classified as a type II tyrosine kinase receptor with two extracellular α-subunits and two transmembrane β-subunits linked by disulfide bonds (1). Stimulation with IGF-1 results in receptor autophosphorylation. The phosphorylated tyrosine residues of the activated receptor can serve as docking sites for the recruitment of certain signaling molecules such as Shc, PI3 kinase, Grb2, Grb10, PLCγ1, and IRS-1 (2). IGFR activation leads to proliferation, differentiation, and inhibition of apoptosis depending on the type of cell (2). IGFR has been shown to play an important role in the establishment and maintenance of the transformed phenotype. IGF-1 and/or IGFR was found to be overexpressed in a variety of human tumors (reviewed in Ref. 3). Mouse embryo fibroblasts with a targeted disruption of the IGFR genes (R^- cells) cannot be transformed by a variety of oncogenes, growth factor receptors, and viral proteins, including v-Ras, Raf, epidermal growth factor receptor, and simian virus 40 T antigen (4–6). Re-expressing IGFR in these R^- cells restores the ability of various oncogenic proteins to transform the R^- cells. Inhibition of IGFR signaling by expressing antisense mRNAs of IGF-1 (7) or IGFR (8, 9), by expressing dominant negative IGFR (10, 11), or by introducing a neutralizing antibody against IGFR (12) has been shown to inhibit the growth of tumor cells in culture and abolish or delay the progression of a variety of tumors in animal models. These studies strongly suggest that overexpression or constitutive activation of IGFR plays an important role in the development of various tumors in humans.

Truncations, internal deletions, point mutations, and amplifications of receptor protein-tyrosine kinases have been shown to lead to their activation and enable them to promote cell transformation and oncogenicity (13). Fusion of PTKs to viral proteins such as Gag has been observed in a great number of naturally isolated oncogenes (14). Complete deletion of the extracellular domain and fusion of the remaining transmembrane and cytoplasmic domains of IGFR to the viral Gag protein of an avian sarcoma virus, UR2, resulted in a constitutively activated and highly oncogenic Gag-IGFR fusion protein that we designated NM1 (Fig. 1*A*) (15, 16). NM1-induced molecular signaling is indistinguishable qualitatively from those of ligand-activated IGFR (15–18). Therefore, NM1 is a suitable model for studying the role of activated or overexpressed IGFR in human malignancy. Expression of NM1 in CEF and NIH 3T3 cells resulted in cell transformation (15, 17, 19). NM1 was also able to efficiently induce tumors *in vivo* (15). In a mutational study where the tyrosines in the cytoplasmic domain of the NM1 Gag-IGFR were systematically mutated to phenylalanines, we found that the mutant Phe-1136 (Y1136F), in which

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[1] The abbreviations used are: IGFR, insulin-like growth factor-I receptor; IGF-1, insulin-like growth factor-I, PI3 kinase, phosphatidylinositol 3′-kinase; PLCγ1, phospholipase C-γ1; IRS-1, insulin receptor substrate-1; CEF, chicken embryo fibroblasts; GEFs, guanine nucleotide exchange factors; dn, dominant negative; ca, constitutively activated; RIE, rat intestinal epithelial; wt, wild type; JNK, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein; aa, amino acid; HA, hemagglutinin; HRP, horseradish peroxidase; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione *S*-transferase; MBP, myelin basic protein.

the third tyrosine of the triple tyrosine cluster within the activation loop of IGFR was replaced with phenylalanine, possessed wild type kinase activity but had reduced transforming potential (17). This result is consistent with the study done in an IGFR knockout cell line (R^- cell) (10). Overexpression of the full-length IGFR with Tyr-1136 mutation in R^- cells could not promote the optimal growth and colony formation of these cells upon IGF-1 stimulation (10). The replacement of tyrosine 950, which serves as the docking site for IRS-1, with phenylalanine did not affect the transforming potential of the Gag-IGFR fusion protein in the CEF system. An additional deletion of 19 aa in the juxtamembrane region of NM1 Gag-IGFR, including tyrosine 950, resulted in a mutant, dS2, that retains kinase activity but has severely reduced transforming ability (17).

Cell transformation is a multifactorial process. Alterations in cell-cell and cell-substratum interactions along with the reorganization of cytoskeletal structures are among the important aspects of malignant cell transformation. The Rho family of small GTPases is an important regulator of cytoskeletal reorganization (reviewed in Ref. 20). These small GTPases cycle between an active GTP-bound state and an inactive GDP-bound state. Their activation is catalyzed by GEFs. GTPase-activating proteins stimulate the weak intrinsic GTPase activity of the GTP-binding proteins, thereby leading to their inactivation. A major function attributed to Rho, Rac, and Cdc42 is the control of the formation of polymerized actin structures and assembly of associated integrin complexes. RhoA activation results in the formation of actin stress fibers and the assembly of focal adhesion complexes (21). Rac1 activation stimulates polymerization of actin at the plasma membrane to induce lamellipodia formation and surface membrane ruffling (22, 23). Cdc42 is believed to regulate the formation of filopodia (24, 25). Nobes *et al.* (23) have established a hierarchy of activation of Rho/Rac/Cdc42 in Swiss 3T3 fibroblasts, wherein Cdc42 activates Rac, which in turn activates Rho. Activated forms of both Rac1 and Cdc42 can induce the formation of focal complexes that contain integrins and focal adhesion molecules.

Growth factors such as platelet-derived growth factor and insulin can stimulate actin polymerization at the plasma membrane to induce lamellipodia formation and surface membrane ruffling in many cell types in a Rac-regulated fashion (23). Vav, a Rac-GEF, has been shown to be regulated by tyrosine phosphorylation and may provide a link between membrane receptors and GTPase activation (26). PI3 kinase appears to function upstream of Rac for the induction of cytoskeletal rearrangements such as membrane ruffling induced by growth factor (23, 27, 28). Regulation of two Rac GEFs, Vav and Sos-1, by the PI3 kinase product, phosphatidylinositol 1,4,5-trisphosphate, is a plausible mechanism of PI3 kinase activation of Rac (29, 30).

In addition to playing a role in cytoskeletal remodeling, Rho GTPases have been shown to play an important role in other cellular processes such as transcriptional activation and cell growth control. They have been shown to be required for G_1 to S cell cycle progression and DNA synthesis (31). Several studies have indicated a role for Rho family GTPases in cell transformation, although these studies have primarily been restricted to those involving Ras (32–35). The role of Rho family GTPases in cell transformation mediated by receptor protein-tyrosine kinases has yet to be defined. It also remains to be established whether the ability of Rho family GTPases to regulate actin re-organization and cell adhesion is necessary for their role in transformation. Additionally, which of the several downstream effectors of Rho family GTPases is required for this role remains to be determined.

In this study we have dissected the known downstream signaling pathways of IGFR, focusing on the GTPases-mediated signaling, in an effort to understand the mechanism of cell transformation mediated by the NM1 Gag-IGFR. Since the kinase-positive, transformation-attenuated mutants, Phe-1136 and dS2, serve as useful tools for identifying substrates that are important for cell transformation induced by NM1, we attempted to identify the downstream signaling pathways that might be impaired in these mutants. Our results suggest that the MAP kinase and the PI3 kinase pathways are required but not sufficient for NM1-mediated cell transformation. Signal transduction pathways mediated by the Rho family GTPases are vital components of the oncogenic IGFR-induced cell transformation. We show that the members of the Rho family GTPases are activated by NM1 and are required for NM1-induced cell transformation. Moreover, our results suggest that Rho appears to play a more important role in promoting escape of contact inhibition, whereas Cdc42 seems to be more important for anchorage-independent growth.

EXPERIMENTAL PROCEDURES

Plasmids and Their Construction—The construction of oncogenic Gag-IGFR (NM1) and NM1 mutants, Phe-1136 and dS2, has been described previously (Fig. 1) (15–17). NM1, Phe-1136, and dS2 were also subcloned into pDEF puro vector, using *Eco*RI/*Bam*HI sites. dn MEK-1 plasmid (pBabe LIDA) was obtained from Dr. Michael Weber (36). pCEV29N-ca Rho/Rac/Cdc42 (RhoAL63, Rac1V12, and Cdc42V12) and dn Rho/Rac (RhoAN19 and Rac1N17) have been described previously (37). dn Cdc42 was obtained from Dr. Alan Hall (38). HA-tagged activated and dominant negative constructs were generated by subcloning each of the constructs into an HA tag containing vector pCEV29HA-JNK (kindly provided by Dr. Andrew Chan at Mount Sinai). The HA-tagged constructs were then transferred into phEF Neo.pGEX-GST-PAK CRIB that contains the p21 (Cdc42/Rac1) binding domain of human PAK1 (aa 70–132) which was a gift from Dr. Bruce Mayer (Howard Hughes Medical Institute/Children's Hospital). pGEX-2TH-GST-ACK42 containing the Cdc42 binding domain of human ACK-1 (aa 504–545) has been described (32). pGEX-4T2-GST-Rok containing the Rho binding domain of Rokα (aa 809–1062) was constructed by polymerase chain reaction amplification of the Rokα cDNA, which was a gift from Drs. T. Leung and Ed Manser of the Glaxo-IMCB Laboratory, Singapore. A 5′-primer (5′-GGGGGATCC**AACACCCTAAAAATGTC-A**-3′) containing a *Bam*HI site and a 3′-primer (5′-GGGCCGGAATTC-**CTTAAGCTCCATATGTAA**-3′) containing an *Eco*RI site were used to amplify the region between aa 809 and 1062 of the Rokα cDNA. The subsequent polymerase chain reaction fragment was cloned in the PGEM®-T vector (Promega). The insert was released using *Bam*HI and *Eco*RI and inserted into the corresponding sites in pGEX-4T2 vector (Promega).

Transfection and Focus Formation Assay—COS-7 cells and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) containing 10% calf serum. COS-7 and NIH 3T3 cells were transfected by calcium phosphate precipitation, as described previously (39). For COS-7 transient transfection assays, cells were transfected at 60–70% cell confluency. Stable transfections and focus formation assay using NIH 3T3 cells have been described previously (19). Briefly, 1.5×10^5 cells were plated in 10-cm tissue culture plates 1 day before transfection. Equal amounts of precipitated DNA were added to two separate plates for each transfecting sample. One of these plates was cultured in DMEM containing 5% calf serum for the focus formation assay, whereas the other plate was selected in medium containing geneticin. The number of drug-resistant colonies formed per plate was determined in order to ensure that equivalent amounts of DNA were utilized and that comparable transfection efficiencies were obtained. Media were changed twice a week following the transfection. Two weeks after transfection, the non-selected plates were fixed, stained with Giemsa dye (Sigma), and photographed for assessing focus formation. The selected plates were enumerated for the drug-resistant colonies, and these colonies were pooled together to generate pooled cultures that were used for further biochemical studies and for soft agar colony formation assay (see below).

Soft Agar Colony Formation Assay—The soft agar assay measuring anchorage-independent growth has been reported elsewhere (19).

Antibodies—Anti-phosphotyrosine RC20 conjugated to horseradish peroxidase (HRP), goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP, and anti-MEK-1 were purchased from Transduction Laborato-

ries. Phospho-p42/44 Erk Thr-202/Tyr-204 monoclonal antibody was obtained from Cell Signaling Technology. Anti-HA monoclonal antibody 12CA5 was purchased from the Mount Sinai Hybridoma Core facility. Anti-IGFR and anti-IRS-1 polyclonal antibody were previously generated in our laboratory (15, 17). Anti-Stat3 and anti-PLCγ1 antibody were purchased from Santa Cruz Biotechnology.

Protein Analysis—Cells were lysed in RIPA buffer, and the equivalent amount of protein was incubated with the respective primary antibodies overnight at 4 °C followed by incubation with protein A-agarose beads (Repligen) for 1 h. The beads were washed three times in the lysis buffer, resuspended in appropriate volume of Laemmli buffer, and subjected to Western blot analysis as described previously (17). The filters were blocked in either 5% milk or 1% bovine serum albumin in TBST buffer (Tris-buffered saline, pH 7.5, 1% Tween 20) and probed with primary antibodies followed by secondary antibody conjugated to HRP in blocking solution and then developed using the enhanced chemiluminescence detection reagent (ECL) (Amersham Pharmacia Biotech).

Kinase Assay—In vitro autophosphorylation, MAP kinase, and PI3 kinase assays were performed as described previously (15, 17).

Immunofluorescence Staining—Parental NIH 3T3 cells were seeded 24 h before transfection. Transient transfection was performed using calcium phosphate transfection method. 24 h later, cells were trypsinized, counted, and seeded onto coverslips placed in 35-mm dishes at the density of 1×10^5 cells/dish. Cells were washed with 1× Hanks' balanced salt solution (Life Technologies, Inc.) and fixed in 2% paraformaldehyde, 0.1% glutaraldehyde, 0.05% Triton X-100 at room temperature for 20 min. After rinsing with 1× Hanks' three times, the coverslips were blocked in 1% bovine serum albumin for 30 min. Cells were incubated with anti-Gag antibody (AMV-3C2, Developmental Studies Hybridoma Bank, Iowa City, IA) for 2 h followed by washing three times with 1× Hanks' before subjecting to incubation with anti-rabbit secondary antibody coupled to fluorescein isothiocyanate along with rhodamine-labeled phalloidin for 1 h. Following three washes in 1× Hanks' solution, the coverslips were mounted on microscope slides using ProLong Antifade Kit (Molecular Probes, Inc.) and examined under the fluorescence microscope.

Assay of Activated RhoA, Rac1, and Cdc42—The assay was performed essentially according to a procedure published previously (33). The synthesis of the fusion polypeptides GST-Rok, GST-ACK, and GST-PAK CRIB encoded in their respective plasmids were induced in *Escherichia coli* with 0.4 mM isopropyl-β-galactopyranoside at 32 °C for 4 h and affinity-purified using 50% slurry of glutathione-Sepharose 4B (Amersham Pharmacia Biotech) according to the Batch Purification method provided by the manufacturer.

COS-7 cells were transiently transfected with phEF HA-tagged wt Rac, wt Rho, or wt Cdc42 along with either the control vector pDEF or pDEF NM1, Phe-1136, or dS2. 24 h post-transfection the cells were starved overnight in serum-free DMEM. Cells were lysed in 1% Nonidet P-40 buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, pH 8.0, 1% Nonidet P-40, 1% Trasylol, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 mM $MgCl_2$). 500 μg of protein lysate from various transfected cells were mixed with 20 μg of GST-PAK CRIB, GST-Rok, or GST-ACK beads and incubated at 4 °C for 2 h. The beads were washed three times in lysis buffer and analyzed by Western blotting to detect the bound GTPases. HA-tagged Rac1, RhoA, and Cdc42 were detected with anti-HA monoclonal antibody 12CA5. The primary antibody was detected with HRP-coupled anti-mouse IgG antibody using ECL.

RESULTS

Characterization of NM1 Gag-IGFR and Its Mutants, Phe-1136 and dS2—We have generated previously an oncogenic variant of IGFR, NM1, which is a Gag fusion receptor protein (Fig. 1*A* and 15, 16). In a mutational analysis of NM1, we identified two loss of function mutants of NM1, Phe-1136 and dS2, that retained kinase activity but were attenuated in their cell transforming ability as tested in primary CEF cells (Fig. 1*A* and see Ref. 17). In order to establish a mammalian cell culture system to study the transformation potential of NM1 and its mutants, Phe-1136 and dS2, we established a stably transfected pool of cells expressing NM1, Phe-1136, and dS2 using NIH 3T3 cells. As shown in Fig. 1*B*, the stable lines express readily detectable levels of the respective Gag-IGFR proteins. Consistent with our previous observation in CEF (17), the Gag-IGFR fusion protein is constitutively active, and the mutant Phe-1136, containing the tyrosine to phenylalanine mutation at position 1136, has comparable levels of *in vitro* receptor autophosphorylation and intracellular tyrosine phosphorylation levels (Fig. 1*B*). The second mutant, dS2, containing a 19-aa deletion in the subtransmembrane region of NM1, spanning amino acids 938–956, has close to wild type receptor autophosphorylation activity and phosphorylation levels (Fig. 1*B*). In order to assess the transforming potential of the NM1 mutants, NIH 3T3 cells expressing NM1 and its mutants, Phe-1136 and dS2, respectively, were examined for their morphological alterations and were assayed for their ability to form colonies in soft agar and foci formation in monolayer. Expression of NM1 in 3T3 cells led to increased mitogenicity (Fig. 1*C*), altered cell morphology (fusiform, elongated, and more refractile cells) (Fig. 1*D*), anchorage-independent growth (Fig. 1*E*), and loss of contact inhibition (Fig. 1*F*). The mutants, Phe-1136 and dS2, showed differential loss of these properties. The mitogenic activity of the NM1 and its mutant receptors was compared by assaying the growth rate of their expressing cells in DMEM containing 0.5% calf serum. The NM1-expressing cells grew significantly faster than the control cells (Fig. 1*C*). The growth rate of dS2- and Phe-1136-expressing cells was indistinguishable from that of the control cells (Fig. 1*C*). Morphologically, NM1- and Phe-1136-expressing cells had similarly altered appearance. dS2-expressing cells, on the other hand, were indistinguishable from the control 3T3 cells (Fig. 1*D*). As observed previously (19), NM1 was able to induce focus formation in NIH 3T3 cells. Phe-1136 retained substantial focus forming ability, 50–60% of NM1 (Fig. 1*F*), but was more severely compromised in its ability to induce anchorage-independent growth (Fig. 1*E*). Under our conditions, NM1-transformed 3T3 cells formed more than 100 colonies, whereas Phe-1136-expressing cells formed only 30 colonies (Fig. 1*E*). dS2 was more severely impaired compared with NM1. dS2-expressing cells displayed almost no focus or colony forming ability (Fig. 1, *E* and *F*).

NM1 Expression Stimulates Activation of Several Downstream Signaling Pathways—To elucidate the signal transduction pathways that mediate IGFR-induced cytoskeletal rearrangements, proliferation, and transformation, we evaluated the level of activation of MAP kinase, PI3 kinase, PLCγ, and Stat3 in the NM1 and its mutant-expressing cells. The Ras/MAP kinase pathway can be activated by the IGFR through the IRS-1-Grb2-Sos and the Shc-Grb2-Sos complex formation (18). The extent of MAP kinase activity in NM1 and its mutant-expressing cells was measured using MBP, as an exogenous substrate (Fig. 2*A*). In addition, the level of MAP kinase activation was assessed by employing a phospho-specific antibody that recognizes only the phosphorylated, active MAP kinase. The level of activated MAP kinase increased in NM1-expressing cells over that of control cells. Comparable levels of MAP kinase activation were seen in the two mutant-expressing cells. The amount of MAP kinase protein immunoprecipitated and the amount of MBP used were similar within the different cell lysates (Fig. 2*A*). In our analysis of the JNK pathway, we found that NM1 expression resulted in a 2–3-fold increase in JNK activity as measured by using GST-Jun as a substrate in an *in vitro* JNK kinase assay. However, both mutants Phe-1136 and dS2 showed comparable levels of JNK activation (data not shown). These results indicate that MAP kinase and JNK activation are not sufficient for the NM1 mutants to promote cell transformation. The requirement of MAP kinase activation for NM1-induced cell transformation was further characterized (see below and Fig. 3).

IRS-1 recruitment followed by its phosphorylation by the receptor creates docking sites for several downstream signaling

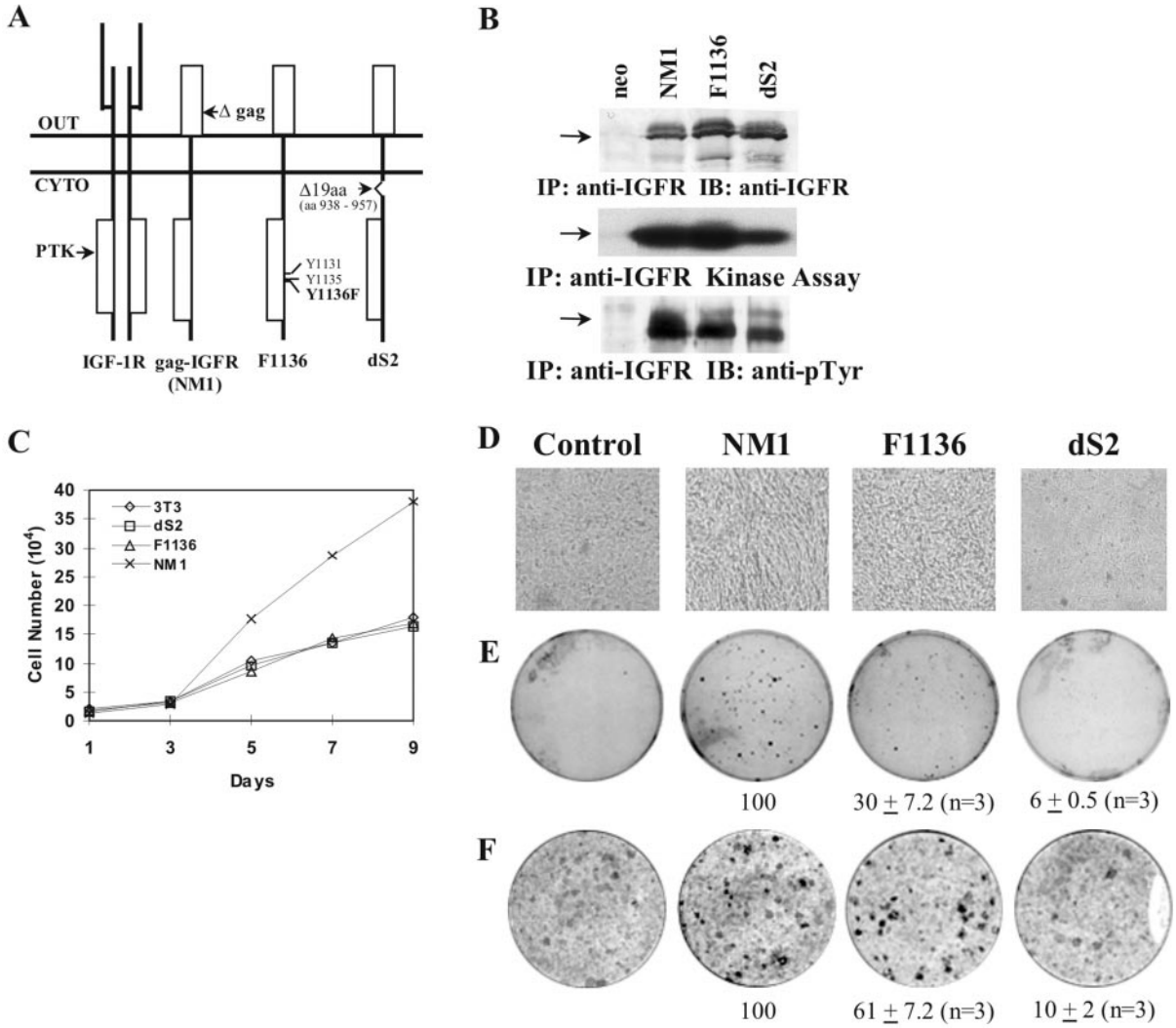

FIG. 1. **Characterization of NIH 3T3 cells expressing NM1 and its mutants, Phe-1136 and dS2.** *A,* schematic representation of full-length IGFR, Gag-IGFR (NM1), and the Gag-IGFR mutants, Phe-1136 and dS2. *OUT,* extracellular; *CYTO,* cytoplasmic; *PTK,* protein-tyrosine kinase domain. *B,* 2 μg of pMX Neo, NM1, Phe-1136, or dS2 was transfected into NIH 3T3 cells and selected with geneticin to generate the pooled cells expressing NM1 or its mutant proteins, respectively, as described under "Experimental Procedures." Cell lysates were prepared, and equivalent amounts were immunoprecipitated (*IP*) with anti-IGFR, subjected to an *in vitro* kinase assay (*middle panel*), and immunoblotted (*IB*) with anti-IGFR (*top panel*) or with anti-pTyr (*bottom panel*). *C,* growth rates of NM1- and mutant-transfected and -expressing cells were compared. 1.5×10^4 cells from each of the pooled cultures of NM1 and mutant receptors were plated per 3-cm dish in duplicate, maintained in DMEM containing 0.5% calf serum, and counted every other day. *D,* photomicrograph of the morphological appearance of cells expressing NM1 and its mutants, Phe-1136 and dS2. *E* and *F,* colony (*E*) and focus (*F*) formation assays were performed as indicated under "Experimental Procedures." The average percentage ($n = 3$) of foci and colony forming ability retained by the mutants Phe-1136 and dS2 as compared with NM1 (which is normalized to 100%) is noted *below* the respective photomicrographs. The colonies (*E*) and foci (*F*) were stained, and photomicrographs were taken 2 weeks later.

molecules, including PI3 kinase (40, 41). The SH2 domain of p85 regulatory subunit of PI3 kinase associates with phosphorylated IRS-1. This association leads to activation of the p110 catalytic subunit of PI3 kinase. The IRS-1 phosphorylation and IRS-1-associated PI3 kinase activity was measured in NM1- and mutant-expressing cells. There was an increase in IRS-1 phosphorylation and IRS-1-associated PI3 kinase activity in the NM1-expressing cells (Fig. 2*B, middle* and *bottom panels,* respectively). This increase was maintained in the single site mutant, Phe-1136, but was altered in the deletion mutant, dS2-expressing cells (Fig. 2*B*). dS2 lacks the IRS-1-docking site, which could explain its reduced IRS-1 tyrosine phosphorylation and loss of PI3 kinase activity. The slightly retarded mobility of IRS-1 protein in dS2-expressing cells is not clear. The potential role of serine phosphorylation in this phenomenon cannot be excluded. This result suggests a requirement for PI3 kinase signaling for NM1-mediated transformation since impairment in PI3 kinase signaling may in part account for the defectiveness of dS2. However, activation of PI3 kinase appears not to be sufficient for cell transformation, since Phe-1136, which is also transformation defective, is still capable of activating PI3 kinase.

Early studies have shown that PLCγ is a substrate of several receptor protein-tyrosine kinases (42). The second messengers generated by PLCγ, inositol 1,4,5-trisphosphate and diacylglycerol, increase intracellular calcium levels and protein kinase C activation, respectively. Overexpression of PLCγ has been shown to lead to malignant transformation of rat fibroblasts (43). The tyrosine phosphorylation levels of PLCγ in the NM1 and mutants-expressing cells were examined (Fig. 3*C*). The phosphorylation level of PLCγ was about 2–3-fold higher in NM1-expressing cells than that of the control cells. In several independent experiments we did not observe any significant differences in the phosphorylation status of PLCγ in the mutant-expressing cells as compared with NM1. There was some variation in the amount of basal level of activation of PLCγ in the several experiments conducted, but the blot presented in Fig. 2*C* represents the overall pattern observed. This result

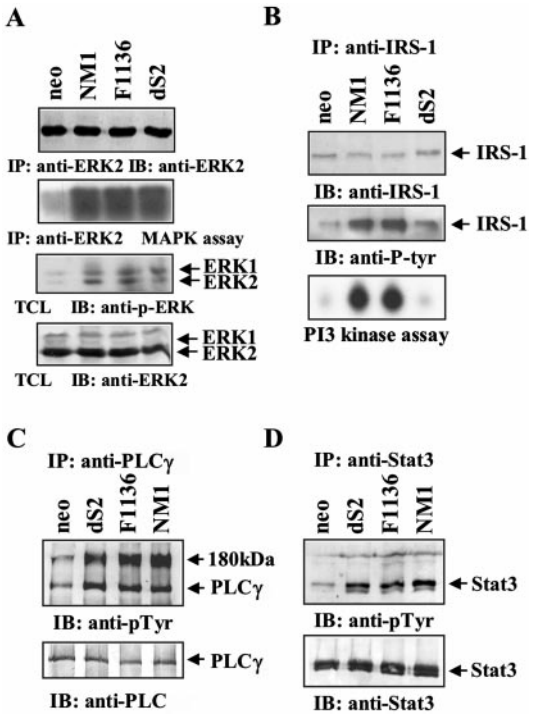

FIG. 2. **NM1 mutants, Phe-1136 and dS2, retain the ability to activate various downstream signaling pathways of NM1.** *A,* cell lysate from each of the stable transfectants was either immunoprecipitated (*IP*) with anti-ERK2 to perform an *in vitro* MAP kinase assay using MBP as an exogenous substrate and immunoblotted (*IB*) with anti-ERK2 to measure protein amount (*top two panels*), or blotted with an activation state specific anti-p42/p44 ERK antibody or anti-ERK2 antibody to quantify ERK activation and expression (*bottom two panels*). *B,* cell lysates were immunoprecipitated with anti-IRS-1 and immunoblotted with anti-IRS-1 (*top panel*) or with anti-pTyr (*middle panel*) or subjected to an *in vitro* PI3 kinase assay (*bottom panel*). *C* and *D,* cell lysates were immunoprecipitated with anti-PLCγ (*C*), anti-Stat3 (*D*), and subjected to immunoblotting with anti-pTyr (*top panels* in *C* and *D*) or anti-PLCγ (*bottom panel* in *C*) or anti-Stat3 (*bottom panel* in *D*). *TCL,* total cell lysate.

indicates that phosphorylation and activation of PLCγ is not sufficient to promote NM1-induced mitogenicity and transformation. A 180-kDa protein was co-immunoprecipitated with PLCγ and was also strongly phosphorylated in dS2, Phe-1136, and NM1 cells (Fig. 2*C*). The identity of this protein remains unknown.

Several groups, including ours (39, 44), have shown recently that Stat3 plays an important role in cellular transformation. We have shown that Stat3 is activated by and required for Ros- and NM1-mediated cell transformation (39). To check if the Stat3 signaling is impaired in the mutants, we assessed the level of Stat3 activation in the mutants-expressing cells. Our results revealed that Stat3 was activated by NM1 and its mutants to a similar level (Fig. 2*D*).

MAP Kinase Activation Is Partially Required for NM1-induced Cell Transformation—To explore further the role of the increased MAP kinase activation observed in NM1-expressing cells, we investigated the effect of a dn MEK-1 (36) on NM1-mediated cell transformation. Stably transfected NIH 3T3 cultures expressing NM1 alone or along with dn MEK-1 (both were pooled cultures derived from over 100 drug-selected colonies) were assayed for their focus and colony forming ability. As shown in Fig. 3, *A* and *B*, both the colony and focus forming ability of NM1 were inhibited in the presence of dn MEK-1. The total number of colonies and, particularly, the size of the colonies were reduced upon dn MEK-1 expression (Fig. 3*A*). There was a 2–3-fold reduction in colony and foci forming ability of NM1 in the presence of dn MEK-1. The expression level of dn

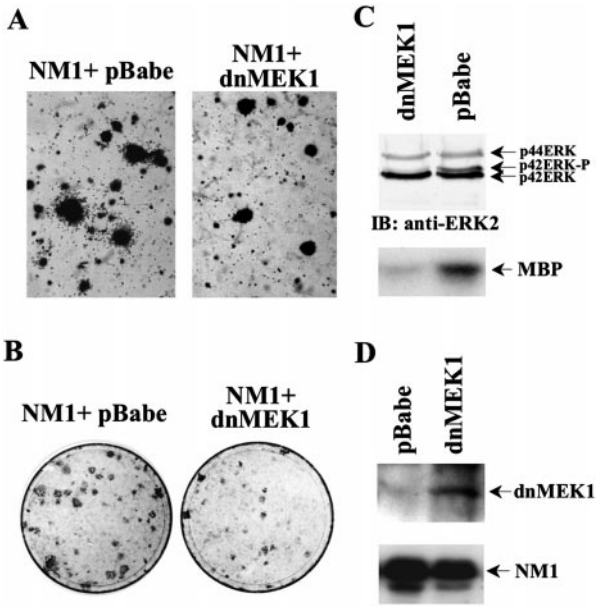

FIG. 3. **Dominant negative MEK-1 partially inhibits NM1-mediated transformation of NIH 3T3 cells.** 3T3 cells were transfected with different plasmids as indicated in the figure. Colony (*A*) and focus (*B*) formation assay were performed as described. *C,* effect of dn MEK-1 on MAP kinase activation was measured by MAP kinase assay as described in *A* (*bottom panel*). 20 μg of total cell lysate was loaded in each lane in a low cross-linking gel for immunoblotting with anti-Erk2 to detect the mobility shift of MAP kinase (*top panel*). *D,* equivalent amount of cell lysates prepared from each stable transfectant was immunoprecipitated with anti-MEK1 or anti-IGFR and subjected to immunoblotting with anti-MEK-1 (*top panel*) or anti-IGFR (*bottom panel*), respectively.

MEK-1 was significantly above that of the endogenous protein (Fig. 3*D*). The activity of MAP kinase was assayed to determine the effectiveness of the dn MEK-1. As shown in Fig. 3*C*, both the mobility shift and the MBP phosphorylation are reduced in NM1 cells expressing dn MEK-1.

Expression of NM1 and Its Mutants Induces Morphological Changes in NIH 3T3 Cells—Growth factors such as insulin and IGF-1 induce actin filament rearrangement in various cell lines, leading to stress fiber breakdown and membrane ruffling (46–48). To study the effect of the expression of NM1 and its mutants on the actin cytoskeleton, the NIH 3T3-expressing NM1 and its mutants were co-stained with an anti-Gag antibody to detect the Gag-IGFR and rhodamine-conjugated phalloidin to visualize F-actin (Fig. 4). Control cells grown in DMEM supplemented with 10% calf serum displayed flat, spread out morphology with parallel actin bundles running across the cells. On the other hand, NM1-expressing cells showed loss of stress fibers, and as a result were less spread. Instead, they were smaller and spindle shaped. In addition, the edges of the cells had various processes resembling microspikes, and a majority of the cells had long filopodia/pseudopod type extension(s) emanating from the cell. The Phe-1136-expressing cells also possessed long filopodia/pseudopod-type extension with pronounced membrane ruffling at the edges (Fig. 4). The dS2 mutant did not seem to display pronounced ruffling, or filopodia/pseudopod-type processes that were observed at the cell periphery in NM1- and Phe-1136-expressing cells (Fig. 4).

Expression of the NM1 Gag-IGFR Induces Activation of Rho, Rac, and Cdc42—To test whether the changes in morphology of NM1 cells reflected an activation of the several Rho family GTPases that are known to be involved in causing actin cytoskeletal rearrangements, we examined whether Gag-IGFR-transfected cells showed any activation (*i.e.* conversion from the GDP-bound form to the GTP-bound form) of RhoA, Rac1,

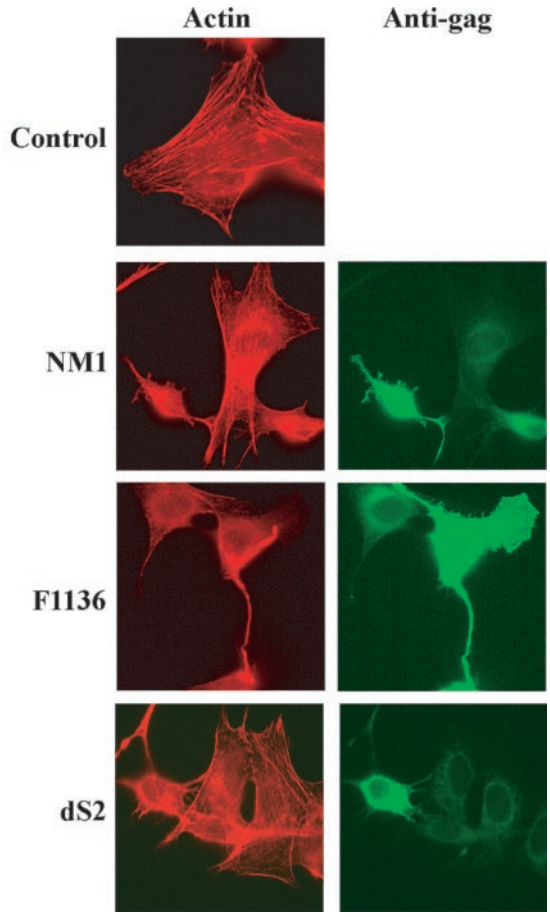

FIG. 4. **The expression of NM1 and its mutants, Phe-1136 and dS2, induces actin cytoskeletal rearrangements.** NIH 3T3 cells were transfected with control, pMX NM1, pMX Phe-1136, or pMX dS2. The transfected cells were co-stained using anti-Gag and phalloidin-tetramethylrhodamine B isothiocyanate as described under "Experimental Procedures." The *left panels* show actin staining, and the *right panels* show anti-Gag staining.

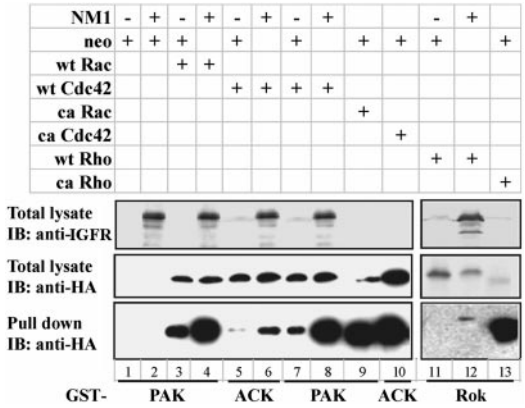

FIG. 5. **NM1 can activate RhoA, Rac1, and Cdc42.** COS-7 cells were co-transfected with different plasmids as indicated in the figure. 24 h post-transfection COS-7 cells were serum-starved overnight and extracted with Nonidet P-40 lysis buffer. 500 μg of total cell lysates were used to perform the pull-down assay as described under "Experimental Procedures." PAK-GST, ACK-GST, and Rok-GST were used to pull down activated Rac (*lanes 3* and *4*), activated Cdc42 (*lanes 5* and *6*), and activated Rho (*lanes 11* and *12*), respectively. PAK-GST was also used to check Cdc42 activation (*lanes 7* and *8*). ca Rac, ca Rho, and ca Cdc42 were used as positive control for PAK-GST (*lane 9*), ACK-GST (*lane 10*), and Rok-GST (13) pull-down assays, respectively. 10 μg of total cell lysates were analyzed by Western blotting using anti-HA to detect GTPase expression or anti-IGFR to detect NM1 expression. *IB*, immunoblotting.

and Cdc42. We used the Rok-, PAK-, and ACK-GST fusion proteins, containing the respective Rho binding domain or CRIB (Cdc42- and Rac-interactive binding) domain, that have previously been shown to bind specifically to the active GTP-bound forms of RhoA, Rac-1, and Cdc42, respectively (25, 33, 49, 50). As shown in Fig. 5, PAK-GST beads bind much more Rac in lysates prepared from NM1-transfected COS-7 cells than the vector-transfected cells, although the expression level of wt Rac was not changed by NM1 expression (compare *lanes 3* and *4*). Similarly, NM1 expression caused increased pull down of Cdc42 by ACK-GST beads as compared with COS-7 cells transfected with empty vector and wt Cdc42 (Fig. 5, *lanes 5* and *6*). Since PAK is a downstream substrate of both Rac and Cdc42, the lysates used in *lanes 5* and *6* were also incubated with PAK-GST, and again, there was more Cdc42 binding to PAK-GST beads when NM1 was expressed (Fig. 5, *lanes 7* and *8*). RacV12 and Cdc42V12 were used as positive controls for the pull-down assay (*lane 9* for PAK-GST and *lane 10* for ACK-GST). By using Rok-GST beads, we show that NM1 can cause activation of Rho (Fig. 5, *lanes 11* and *12*). RhoL63 was used as a positive control (Fig. 5, *lane 13*). These results clearly indicate that NM1 Gag-IGFR induces activation of Rac1, Cdc42, and RhoA, although with different potency.

The Mutants, Phe-1136 and dS2, Are Attenuated in Their Ability to Activate Rho, Rac, and Cdc42—To investigate whether the loss-of-function mutants, Phe-1136 and dS2, can activate Rho family GTPases, we performed similar pull-down assays as described for NM1 (Fig. 5). Similar co-transfection experiments were conducted in COS-7 cells. NM1, Phe-1136, or dS2 expression vectors were co-transfected with either wt Rac, wt Cdc42, wt Rho, or control vector. For comparing the level of Rac activation, PAK-GST beads were incubated with lysates prepared from the various transfected cells. Phe-1136 and dS2 could activate Rac as potently as NM1 (Fig. 6*A*). By using Rok-GST beads to measure Rho activation, we observed that as compared with NM1, Phe-1136 retained substantial capability in inducing Rho activation, but dS2 did not lead to any significant activation of Rho over the basal level. The ACK-GST pull down to ascertain Cdc42 activation in the various receptor-expressing cells revealed that Cdc42 is activated by NM1, and this activation is attenuated in Phe-1136 and further reduced in dS2-expressing cells (Fig. 6*A, right panel*). All results were normalized for protein levels of the various GTPases as well as NM1 and its mutant proteins, and the data are presented in the form of a histogram (Fig. 6*B*).

Colony and Focus Forming Ability of NIH 3T3 Cells Expressing NM1 Is Differentially Inhibited by Rho Family GTPases—Since Rac, Rho, and Cdc42 were activated by NM1, we investigated whether these small GTPases were required for NM1-mediated transformation. NIH 3T3 cells were co-transfected with NM1 together with control vector or dn mutants of Rho/Rac/Cdc42 (RhoAN19, Rac1N17, Cdc42N17, respectively). The focus forming ability (Fig. 7*A*) of NM1 was inhibited to various extents by the dn GTPases. dn Rho was most potent at inhibiting the focus forming ability of NM1, reducing it by about 86% ($n = 3$) (Fig. 7*A*). dn Rac1 and dn Cdc42 resulted in ~50 and 20% ($n = 3$) inhibition, respectively (Fig. 7*A*). The dn GTPases also inhibited the colony forming ability but differentially. dn Cdc42 inhibited colony formation by ~70%, dn RhoA by ~60%, followed by dn Rac1 at ~40% ($n = 3$) (Fig. 7*B*). The NM1 and dn Rho or dn Cdc42 co-transfectants showed both a decrease in colony number and size as compared with the co-transfection of NM1 with the control vector (data not shown). These results indicate that Rho family GTPases are required for NM1-induced transformation of 3T3 cells.

The dominant negative mutants of Rho family GTPases work

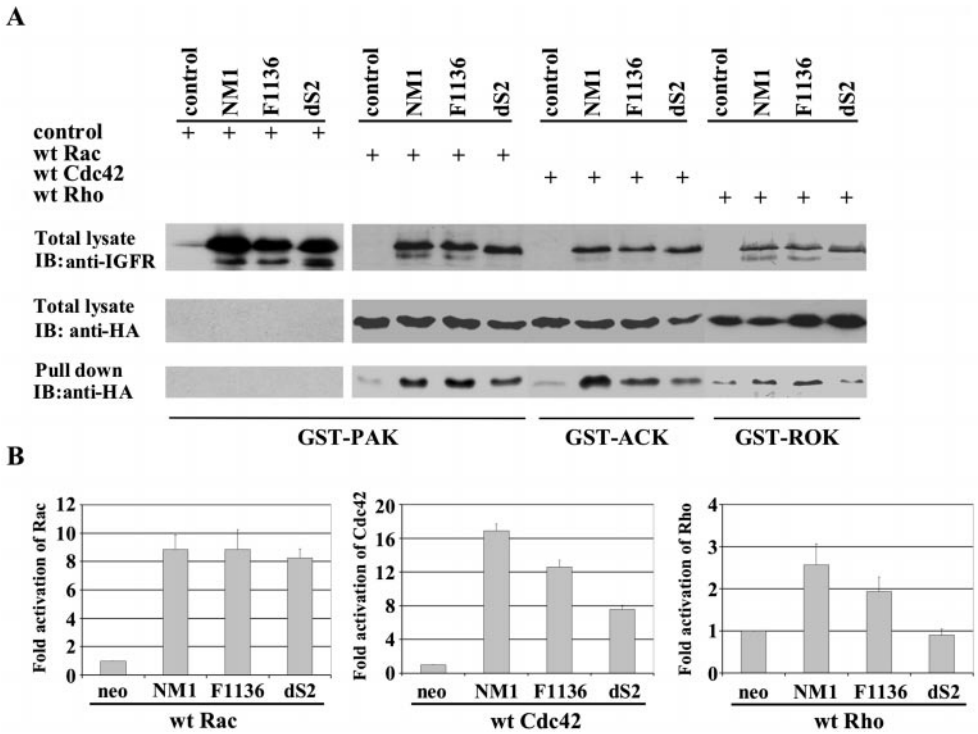

FIG. 6. **NM1 mutants, Phe-1136 and dS2, have reduced ability to activate Rho family GTPases.** *A,* COS-7 cells were co-transfected with different plasmids as indicated in the figure, and pull-down assays were performed as described in Fig. 5 (*bottom panel*). Protein expression levels were detected using anti-IGFR (*top panel*) and anti-HA (*middle panel*). The blot shown represents typical expression levels. *B,* the data were normalized based on protein expression levels of both the GTPases and NM1 proteins. The values for the intensity of the protein bands were calculated using Molecular Dynamics, ImageQuant Software. The fold activation of the GTPases was calculated by dividing the value of the pull-down band by the product of the value for the NM1 (or the mutants) and the value for the given GTPase protein band. The *histogram* represents the average of three independent experiments. *IB*, immunoblotting.

by sequestering GEFs and thereby inhibiting the function of the endogenous GTPase proteins. Since the Rho family GTPases share several common upstream activators and downstream effectors, the specificity of the dominant negative mutants may be questioned. Parallel studies that utilize alternative inhibitors of Rho family GTPases would independently confirm our results obtained with the dominant negative mutants. C3 transferase is a toxin produced by the bacterium, *Clostridium botulinum*, that specifically ribosylates asparagine 41 (Asn-41) within the RhoA effector-binding site domain, thereby inhibiting its interaction with downstream substrates. C3 transferase is therefore considered a specific inhibitor of RhoA. Co-expression of C3 transferase and NM1 resulted in a 60–70% inhibition of NM1-induced colony formation, confirming the inhibition seen with dn RhoA (Fig. 7*B*). Similarly, we used an expression vector encoding ACK42, a 42-amino acid fragment of the Tyr kinase ACK-1 that specifically binds GTP-bound Cdc42, to inhibit Cdc42 (32). Co-expression of ACK42 and NM1 resulted in a 75% inhibition of NM1-induced colony formation (Fig. 7*B*). These inhibitors independently confirmed the results obtained with the dn mutants of GTPases that Rho family GTPases signaling are required for NM1-induced cell transformation.

The Transforming Ability of Mutants, Phe-1136 and dS2, Is Differentially Rescued by Rho, Rac, and Cdc42—NM1 Gag-IGFR could induce activation of Rho, Rac, and Cdc42, whereas the two mutants, Phe-1136 and dS2, were attenuated in such ability. Additionally, NM1-induced NIH 3T3 cell transformation was inhibited by dn Rho family GTPases. We next investigated whether co-expression of activated mutants of Rho, Rac, and Cdc42 could rescue the transforming ability of the mutants, Phe-1136 and dS2. The expression plasmids for RhoAL63, Rac1V12, and Cdc42V12 were transfected with control vector alone to determine their basal level of focus forming activity. The activated Rac and Cdc42 had very low or no focus forming activity (2 and 0 foci per μg, respectively) (data not shown). Activated Rho had a basal level of foci (12 foci per μg) (data not shown). Co-expression of mutant Phe-1136 along with activated RhoA resulted in a 2.5-fold increase in the focus forming ability of Phe-1136 as compared with Phe-1136 expression with control vector (50 $\pm$ 11 foci per μg) (Fig. 8*A*). This approximates the focus forming ability of NM1. Co-expression of dS2 along with activated RhoA resulted in a 9.5-fold increase in the focus forming ability of dS2-expressing cells as compared with co-expression of dS2 with control vector (7 $\pm$ 2 foci per μg) (Fig. 8*B*). This is ~60% of the focus forming ability of NM1. This was not surprising since Phe-1136 retains substantial focus forming ability, whereas dS2 is severely impaired in such (Fig. 2*F*). Additionally, dS2 expression could not induce Rho activation (Fig. 6*A, middle panel*, and Fig. 6*B, middle*). Among the three activated Rho family GTPases used, the overall results suggest that activated Rho was most potent in rescuing the focus forming ability of the mutants.

The anchorage independent growth of Phe-1136-expressing cells was increased by 1.6-, 2.4-, and 4.4-fold by Rac1V12, RhoAL63, and Cdc42V12, respectively, as compared with co-expression of Phe-1136 with the control vector (Fig. 8*C*). Similarly, NIH 3T3 cells co-expressing dS2 with Rac1V12, RhoAL63, and Cdc42V12 showed a 1.2-, 2.2-, and 4.1-fold increase, respectively, in their colony forming ability as compared with co-expression of dS2 with the control vector (Fig. 8*D*). The expression levels of the constructs from various transfectants are shown in Fig. 8*E*. These results suggest that ca Cdc42 appears to be more important for anchorage-independent growth. Independently, we have generated RIE cell lines expressing NM1, Phe-1136, and dS2. As observed in the 3T3 cell

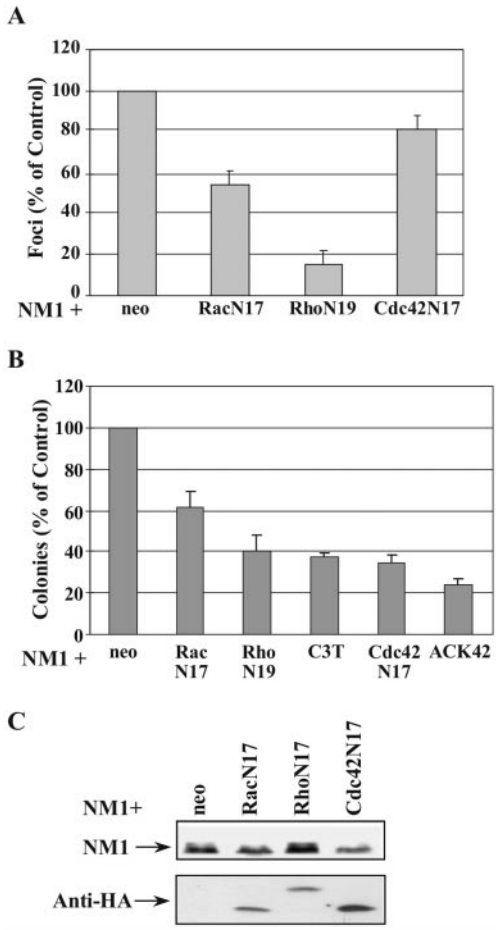

FIG. 7. **NM1-induced cell transformation is inhibited by dn Rho family GTPases.** NIH 3T3 cells were co-transfected with the different plasmids as indicated in the figure. Focus (*A*) and colony (*B*) formation assays were performed as described under "Experimental Procedures." The foci or colonies were stained and counted, and the results were plotted as a histogram representing the average of three independent experiments. *C,* protein expression levels were detected using anti-IGFR (*top panel*) and anti-HA (*bottom panel*). *FFU,* focus-forming units; *CFU*, colony-forming units.

system, the mutants, Phe-1136 and dS2, are impaired in their colony forming ability in the RIE cells as well (data not shown). In rescue experiments performed (in RIE cells) using ca Rac/Rho/Cdc42, we confirmed that ca Cdc42 and to a lesser extent ca Rac were able to enhance the colony forming ability of the mutants, Phe-1136 and dS2, whereas ca Rho expression had no significant effect on the colony forming ability of the mutants.

In order to ascertain that the rescue of the mutants, Phe-1136 and dS2, was a result of a restoration of a signaling pathway(s) that is defective in the mutants, and not just a parallel pathway that is being activated by the activated GTPases, we expressed the wild type NM1 Gag fusion receptor along with ca Rac/Rho/Cdc42 and performed colony and focus assay experiments. We did not observe a dramatic increase in the number of colonies and foci induced by NM1 in the presence of the activated GTPases with no more than a 1.25-fold increase in the focus and colony forming ability of NM1 in the presence of the various ca GTPases (Table I). There was a significant increase in the size of the colonies in the presence of NM1 and ca Rac or ca Cdc42 but not with ca Rho (data not shown). There was a modest increase in the number of foci formation accompanied by an increase in the size of the foci induced by NM1 in the presence of ca Rho but not with ca Rac and ca Cdc42.

The ability of Rho to accentuate the focus forming ability of cells was observed independently using the full-length IGFR. IGF-1 stimulation of cells that had been co-transfected with full-length IGFR along with ca Rho resulted in a dramatic increase in the size of the foci induced by ca Rho as compared with the foci induced by ca Rho in the absence of ligand. The number of foci that were greater than 0.8 mm in size increased by 3.5-fold in the presence of IGF-1 stimulation (data not shown). ca Rac and ca Cdc42 failed to promote any significant foci in the presence or absence of IGF-1. Also, expression of ca Rac and ca Cdc42 but not ca Rho resulted in an increase in the size and number of colonies formed in the presence of IGF-1 by an IGFR-overexpressing cell line (data not shown).

Taken together, our results indicate that Rho/Rac/Cdc42-mediated signaling is important for NM1- and IGFR-induced focus and colony formation. Moreover, Rho appears to play a more important role in promoting escape of contact inhibition, whereas Cdc42 is more important for anchorage-independent growth.

DISCUSSION

IGF-1- and IGFR-mediated signaling has been shown to play an important role in inducing cell proliferation, rearrangement of the cytoskeleton, cell adhesion, induction, and/or maintenance of the transformed phenotype. The role of Rho family GTPases in IGFR-mediated cell transformation has not been explored. By using a constitutively activated IGFR variant, NM1, we have shown that Rho family GTPases are activated by IGFR and are differentially required for IGFR-mediated cell transformation. Our data suggest that Rho-mediated signaling is more important for abrogation of contact inhibition and that of Cdc42 is more important for anchorage-independent growth.

Constitutive activation of the MAP kinase/Erk pathways has been associated with promotion and/or maintenance of the transforming and tumorigenic phenotype (51–53). We found that MAP kinase was activated in NM1-expressing cells and was at least partially required for NM1-induced cell transformation in NIH 3T3 cells. The NM1 mutants, Phe-1136 and dS2, are fully capable of inducing MAP kinase activation, suggesting that in NIH 3T3 cells activation of MAP kinase, although partially required, is not sufficient for the oncogenic IGFR-induced cell transformation. MAP kinase activation was found to be dispensable for v-*src*-induced cell transformation of Rat2 fibroblasts by Aftab *et al.* (54). In addition, inhibition of MAP kinase- or PI3 kinase-mediated signaling alone was not found to be sufficient to block v-*src*-induced cell transformation of CEF, although inhibition of MAP kinase by PD98059 did result in 40% inhibition of the colony forming ability of v-*src* (55). Simultaneous inhibition of both MAP kinase and PI3 kinase substantially blocked v-*src*-induced cell transformation (55). IGFR-mediated cell transformation has also been shown to have a Ras-independent and a PI3 kinase-independent pathway (5, 56). The apparent differences in the requirement of MAP kinase activation for various oncogenic protein-mediated cell transformation may reflect types of cells and oncogenes used in the individual studies.

IRS-1 is an important substrate for IGFR and IR signaling and is shown to regulate the mitogenic response of IR (57). NM1 mutant, dS2, containing a 19-aa deletion in the juxtamembrane region, including the binding site of IRS-1, Tyr-950, is severely impaired in its transforming ability. Expression of NM1 and Phe-1136 but not dS2 induces increased IRS-1 tyrosine phosphorylation. This is correlated with a loss of IRS-1-associated PI3 kinase activity in the dS2-expressing cells. It has been suggested that PI3 kinase can associate directly with IGFR through tyrosine 1316 in the carboxyl terminus of IGFR (58). Whether the IGFR-associated PI3 kinase activity is also affected in dS2 was not ascertained. Inhibition of PI3 kinase

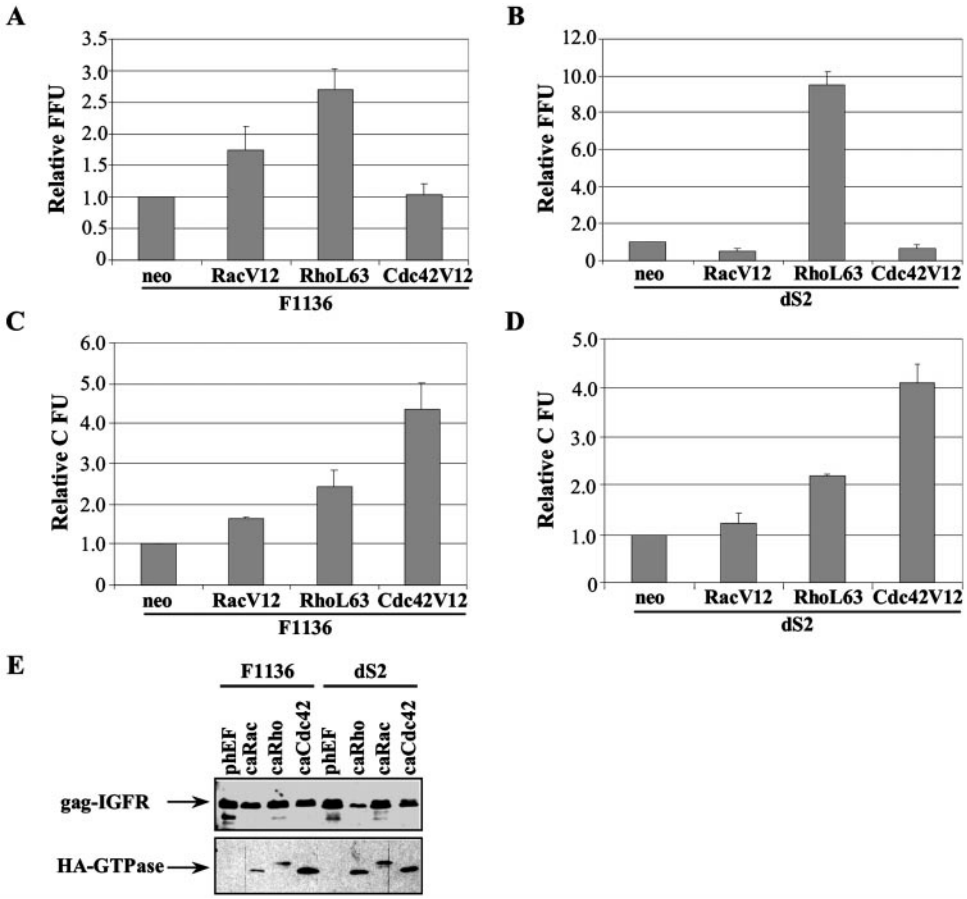

FIG. 8. **Constitutively activated Rho family GTPases can rescue the transforming ability of NM1 mutants, Phe-1136 and dS2.** NIH 3T3 cells were co-transfected with the indicated plasmids. Focus (*A* and *B*) and colony (*C* and *D*) formation assays were performed as described under "Experimental Procedures." The foci or colonies were stained and counted, and the results were plotted as a histogram representing the average of three independent experiments. *E,* protein expression levels were detected using anti-IGFR (*top panel*) and anti-HA (*bottom panel*).

TABLE I
NIH 3T3 cells were co-transfected with NM1 and either control vector or ca Rac, ca Rho, or ca Cdc42

Focus and colony formation assays were performed and enumerated as described. These results are representative of two independent experiments. CFU indicates colony-forming units; FFU indicates focus-forming units.

Effect of ca Rac/Rho/Cdc42 on NM1-induced foci and colony formation		
NM1 and ca GTPases	% CFU	% FFU
NM1 + neo	100	100
NM1 + ca Rac	123	103
NM1 + ca Rho	100	125
NM1 + ca Cdc42	120	106

pathway using a dominant negative mutant of PI3 kinase or a PI3 kinase pharmacological inhibitor, LY294002, resulted in 60–70% inhibition of NM1-mediated cell transformation (data not shown). These results suggest that IRS-1-mediated activation of PI3 kinase is likely an essential pathway but is not sufficient for NM1-induced cell transformation since Phe-1136 is attenuated in cell transformation despite its ability to activate PI3 kinase.

Stimulation of IGFR has been shown to lead to actin cytoskeletal rearrangements and cell motility in several cell types (59–61). It has long been known that transformation of mammalian cells by oncogenes such as v-*src* results in actin cytoskeletal rearrangements and disintegration of stress fibers and adhesion plaques (62, 63). Insulin stimulation of rat fibroblasts overexpressing the insulin receptor leads to a PI3 kinase-mediated initial breakdown of stress fibers followed by transient membrane ruffling and return of stress fiber polymerization (48). In our staining of IGF-1-treated and -untreated NIH 3T3 cells overexpressing human IGFR, the control cells were indistinguishable from normal 3T3 cells, whereas the treated cells showed an elongated cell body with long filopodial extensions and fine microspikes along the cell edges (data not shown). Taken together, these results indicate that IGF-1 stimulation leads to distinct cytoskeletal rearrangements. NM1- and Phe-1136-expressing cells display cell surface processes, microspikes, and filopodia/pseudopod type structures. Phe-1136 retains the ability to activate PI3 kinase and partial ability to activate Cdc42 and Rho which may explain the cytoskeletal rearrangements observed upon its expression in 3T3 cells. dS2 expression does not lead to formation of cell surface processes, although cells appear smaller and rounded like NM1-expressing cells. dS2 is impaired in promoting IRS-1 tyrosine phosphorylation and IRS-1-associated PI3 kinase activity; it is also impaired in its ability to activate Rho and Cdc42. These differences between NM1 and dS2 may account for their distinct effects on cell morphology in their respective expressing cells.

Activation of Rho family GTPases by growth factor receptors has been inferred from the appearance of specific cytoskeletal changes. Recently, biochemical pull-down assays have been developed to monitor the direct activation state of Rac and Cdc42. These assays take advantage of the specific binding of the downstream effectors of Rho/Rac/Cdc42 to GTP- but not GDP-loaded GTPases (25, 33, 49, 50). We used PAK-GST, ACK-GST, and Rok-GST to determine whether NM1 expression can lead to activation of Rho, Rac, and Cdc42. Our results show

that indeed the constitutively activated Gag-IGFR can lead to activation of these Rho family GTPases. In addition to their role in cytoskeletal remodeling, Rho family GTPases have been shown to be required for Ras-mediated transformation, and activated forms of the GTPases cooperate with Raf in focus formation assays in NIH 3T3 cells (31–35). Additionally, several GEFs for Rho family members have been found as oncoproteins (reviewed in Ref. 64). NM1-induced cell transformation is dependent on members of Rho family GTPases. Rescue experiments using Rac1V12, RhoAL63, and Cdc42V12 showed that the Rho was able to rescue the focus forming ability of dS2 to a greater extent. This correlates with the inability of dS2 to activate Rho in the biochemical pull-down assays. Phe-1136, on the other hand, retains considerable focus forming activity (60% of NM1). Phe-1136 also retains significant Rho activation thus further suggesting that Rho plays a more important role in focus formation. On the other hand, ca Cdc42 was more effective in restoring the ability of the mutants Phe-1136 and dS2 to induce anchorage-independent growth. Both dS2 and Phe-1136 are impaired in their ability to activate Cdc42, particularly dS2, as measured by the pull-down assays.

Clearly the defectiveness of dS2 in activating GTPases cannot account entirely for its decreased focus and colony inducing ability since it is also impaired in the activation of PI3 kinase which is also important for NM1-induced cell transformation as demonstrated by using a dominant negative mutant of PI3 kinase and its pharmacological inhibitors, wortmannin and LY294002.[2] This is consistent with the finding that neither ca Rho nor ca Cdc42 was able to restore the focus and colony forming ability of dS2 to the level of NM1. The mutant Phe-1136 has no detectable defectiveness in the IGFR-mediated signaling pathways analyzed thus far and displays only partial impairment in the activation of Rho and Cdc42 that may account for its partial defectiveness in cell transformation, especially colony formation. Whereas ca Rho was able to restore the focus forming ability of Phe-1136 to the level of NM1, neither ca Rho nor ca Cdc42 was able to restore the colony forming ability of the mutant to the NM1 level, particularly with respect to the size of the colonies. Other IGFR-induced signalings such as integrin-mediated pathways need to be examined to explore further the molecular basis for its defectiveness in transformation.

To our knowledge, this is the first report that differentiates the role of Rho family GTPases in distinct properties of transformed cells. The ability of Rho to accentuate the focus forming ability of cells suggests that Rho expression may result in the abrogation of a negative signal resulting from close contact of cells. There is some evidence that Rho plays a role in regulating cellular $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ levels (65, 66). It was shown that Rho activity is required to suppress the activated Ras-induced $p21^{Waf1/Cip1}$ levels and allow G_1 to S phase progression (66). This could be a possible mechanism for Rho-mediated escape of contact inhibition. Similarly, signaling through Cdc42 could result in positive signals for anchorage-independent growth. Cdc42 expression results in increased MAP kinase activation in anchorage-independent conditions (67). These small GTPases may control different transformation properties of cells by being discriminatory in their interaction with specific downstream effectors. Specific downstream effectors of each of the Rho family members may be responsible for the distinct roles of these GTPases in regulating cellular functions. Rho-specific effectors, ROCK family of kinases have been suggested to be important for the stress fiber formation, serum response factor activation, and the focus formation ability of Rho (68, 69). The WASP family of proteins and POR1 have been suggested as the specific downstream effectors for Cdc42- and Rac-mediated cytoskeletal rearrangements, respectively (reviewed in Ref. 70). The specific downstream effector(s) and downstream signaling pathways of Rho family GTPases that are responsible for their role in cell transformation have not been clearly defined.

In summary, the results presented here, show that Rho family GTPases play an important role in IGFR-mediated cell transformation. The MAP kinase, PI3 kinase, and Stat3 signaling pathways are also required to various degrees for IGFR-mediated cell transformation, but their activation, individually or in combination, is not sufficient. NM1 can induce activation of Rho, Rac, and Cdc42, and inhibition of their endogenous counterparts leads to inhibition of NM1-mediated cell transformation. Rho-mediated signaling promotes the cells to escape contact inhibition, whereas Cdc42-mediated signaling promotes the cells to grow in anchorage-independent conditions.

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[2] P. Sachdev, Y.-X. Jiang, W. Li, T. Miki, H. Maruta, M. S. A. Nur-E-Kamal, and L.-H. Wang, unpublished observations.

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